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Finding new potential antimicrobials in natural environments: Do bacterial isolates from ancient and modern woodland, and farmland show the ability to produce inhibitory compounds?

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WARRANTY STATEMENT

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Abstract

Research and development into the production of novel antimicrobials is greatly needed due to the current global threat that antimicrobial resistance is presenting. In the present study, modern and ancient forestlands, Friston Forest, (Sussex, UK), and New Forest, (Hampshire, UK), and Dawes Farm, (Warnham, West Sussex, UK) were sampled in the hope of isolating organisms with inhibitory activity and/or similar morphology to that of the *Streptomyces* spp. for further assessment of antagonistic activity against selected test bacteria. Perpendicular screening, and the newly developed starvation method, were used to characterise the inhibitory ability of organisms isolated against sensitive test strains of *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, and *Proteus mirabilis*. Organisms displaying inhibitory activity towards sensitive organisms were further tested using clinically isolated test organisms from the same genus. Perpendicular screening confirmed 11 (15.9%), 5 (14.7%), and 13 (30.2%) of the total organisms isolated from Friston Forest, New Forest and Dawes Farm respectively, with inhibitory activity towards one or more of the test organisms used, with eight of these displaying further inhibition of at least one of the clinical test organisms used in this method. The starvation method, used for further antibacterial screening, involving the incubation of the potential antimicrobial producing organisms in minimal media for long incubation periods, confirmed 21 (30.4%) 12 (35.3%), and 9 (20.9%) isolates from Friston Forest, New Forest, and Dawes Farm vets with inhibitory ability.

All organisms isolated were tested using Gram staining, with those displaying resemblance to the *Streptomyces* spp. selected for further determinative identification using the polymerase chain reaction, with primers specific to the 16S rRNA gene of the *Streptomyces* genus. Nineteen isolates were determined as belonging to this genus, with 42.1% of the confirmed *Streptomyces* spp. displaying inhibitory activities.

The same soil samples were used for the cultivation of potentially pathogenic bacteria, with biochemical testing used to determine the identity of each of the 222 organisms isolated, to a genus level. Across the three locations three isolates were identified as belonging to *Salmonella* spp., 21 as *Staphylococcus aureus*, nine *Pseudomonas* spp., two *Staphylococcus epidermidis*, four *Klebsiella* spp. and four *Escherichia coli*. The screening carried out in this study confirmed the ability for the isolation of pathogenic organisms as well as soil organisms with antibiotic producing capability, from the previously untested farm and forestland sampled.

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AMR – antimicrobial resistance

APO – antimicrobial producing organisms

ARG – antibiotic resistance genes

CDC – Centres for Disease Control and Prevention

CRE – carbapenem-resistant *Enterobacteriaceae*

ECDC - European Centre for Disease Prevention and Control

ESBLs – Extended Spectrum Beta Lactamases

EUCAST- European Committee on Antimicrobial Susceptibility Testing

HGT – horizontal gene transfer

MDR – multiple drug resistance

MRSA – methicillin-resistant *Staphylococcus aureus*

PCR – polymerase chain reaction

PDR – Pandrug-resistant

TBE – tris-borate-ethylenediametetraacetic acid

TLC – thin-layer chromatography

VRE – vancomycin-resistant Enterococci

VRSA – vancomycin-resistant *Staphylococcus aureus*

WHO – World Health Organisation

XDR – extensively drug-resistant

1.0 Introduction

1.1 Antibiotic resistance

Antimicrobials cover a wide range of natural as well as synthetic agents that can be used to combat infections in both humans and animals, which are often produced by microorganisms themselves, primarily to better their niche exploitation in their given environment (Van Hoek *et al.,* 2011; Hibbing *et al.,* 2010). Antibiotics are one example of an antimicrobial, defined as a natural substance produced itself by a microorganism, with the ability to kill or inhibit the growth of other microorganisms (CDC, 2017). However, these antimicrobial drugs are increasingly being classified as ineffective for the treatment of various microorganisms, due to the ability of these resistant pathogens to overcome the action of particular antimicrobials. This increasing resistance has caused the development of multiple drug resistant strains of certain organisms, including those with resistance mechanisms towards several of the current last line antibiotics, presenting a major global, and indeed, 'one health' concern (WHO, 2014). In some pathogens, resistance has been observed against the carbapenem class of antibiotics by carbapenem-resistant *Enterobacteriaceae* (CRE), which has been linked with high mortality in human infections (>50%) (Zarkotou *et al.,* 2011). Vancomycin-resistant *Staphylococcus aureus* (VRSA) has also been isolated, with vancomycin being one of the last resort treatments for serious infections caused by Gram-positive bacteria (Hu *et al.,* 2016; Rice, 2006; Whitener *et al.,* 2004). As well as this, resistance towards the polymyxin group of antibiotics, including colistin, has been seen in recent years, by organisms belonging to the *Enterobacteriaceae* group (Laxminarayan *et al.,* 2013; Olaitan *et al.,* 2014). These polymyxins are used as the last line of defence against infections, caused by pan-drug resistant Gram-negative organisms, hence maintaining the effectiveness of these drugs is crucial (Gao *et al.,* 2016; Olaitan *et al.,* 2014). This emergence and increasing frequency of resistant bacteria is apparent all over the world, with it currently causing around 700,000 deaths every year, which is estimated to increase to 10 million deaths in 2050 if antimicrobial resistance (AMR) is not tackled (O'Neill, 2016).

Antimicrobial resistance (AMR), defined by the Centres for Disease Control and Prevention (CDC), and European Centre for Disease Prevention and Control (ECDC), using the displayed effectiveness of several drugs against bacterial strains (Figure 1.1 and Table 1.1), can occur in multiple ways allowing bacteria and other microbes to overcome the action of an antimicrobial, making it no longer effective (Magiorakos *et al.,* 2011). *De novo* mutations in microbes can directly affect targets or pathways targeted by antimicrobials, or cause the upregulation of efflux pump mechanisms or enzymes that affect the antimicrobial's structure, providing an advantage for the mutated strain, enabling the organism to overcome the action of particular antimicrobials (Laxminarayan *et al.,* 2013; Levy, 1992; Török *et al.,* 2012). The acquisition of these mutations conferring resistance (resistance genes) can also be, in some cases, transmitted between the same or different bacterial species (Von Wintersdorff *et al.,* 2016). This often involves the use of plasmids or other mobile genetic elements in horizontal gene transfer (HGT), allowing resistance to spread

and hence increasing the prevalence of particular resistance genes (Török *et al.,* 2012; Mazel and Davies, 1999).

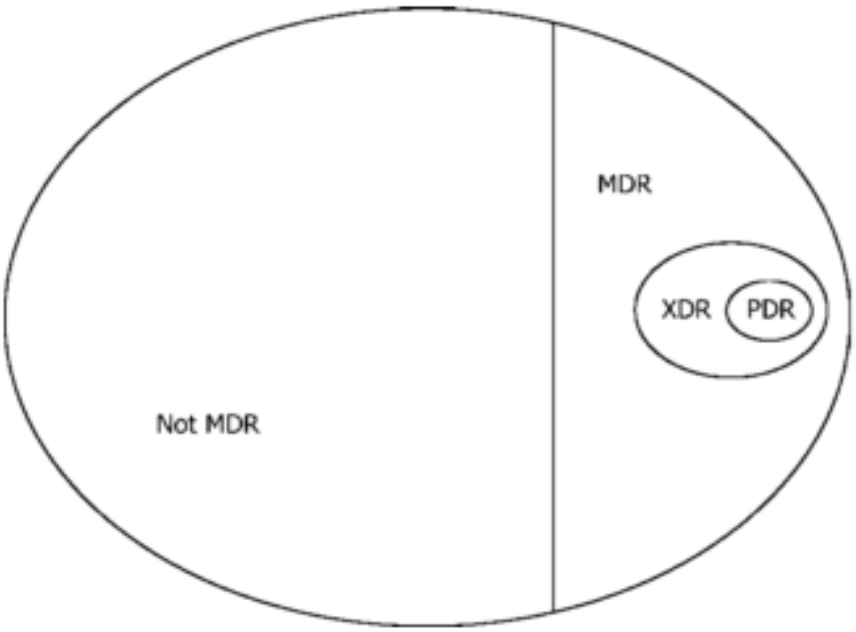

Figure 1.1: The relationship between the terms Multidrug-resistant (MDR), Extensively drug-resistant (XDR) and Pandrug-resistant (PDR) used to define the drug sensitivity of specific bacterial organisms (taken from Magiorakos *et al.,* 2011). An example of these definitions of antimicrobial resistance is given for *Pseudomonas aeruginosa* in Table 1.1.

However bacterial resistance, due to naturally occurring antibiotic resistance genes (ARG), is an ancient mechanism that has occurred in bacteria before antibiotics were of major use in the medical industry. Perron and colleagues (2015) demonstrated that bacteria samples taken from ancient permafrost soils harboured several different resistance genes that conferred resistance to such antibiotic classes as the β-lactams and tetracyclines, as well as the non-naturally occurring antibiotic amikacin, with great genetic similarity to those resistance genes seen in pathogenic bacteria.

Although AMR has been seen to be developed amongst organisms in natural ecosystems, it is clear that exaptation and evolution leading to the development of resistant organisms, which have undergone favourable *de novo* mutations or acquired ARG, is the consequence of selective pressure, created by the vast number of antimicrobials in use in recent years (Martinez, 2012; Mazel and Davies, 1999; Rice, 2006; Wellington *et al.,* 2013). With the use of HGT, these now advantageous genes can be passed between bacteria, including different strains and species, allowing resistance genes to spread and be maintained, while rendering certain antibiotics ineffective (Laxminarayan *et al.,* 2013).

Table 1.1: The antimicrobial susceptibility profile of *Pseudomonas aeruginosa* that fits the definitions used by the European Centre for Disease Prevention and Control (ECDC) and the Centres for Disease Control and Prevention (CDC). Multidrug-resistant (MDR) is defined as non-susceptibility to at least one antimicrobial in three or more classes/categories. Extensively drug-resistant (XDR) is defined as susceptibility towards only two classes/categories of antimicrobials (or non-susceptibility to at a minimum of one agent in all but two categories). Pandrug-resistance (PDR) is defined as a strain that is no longer susceptible to all agents in all antimicrobial classes/categories (Magiorakos *et al*., 2011). The relationship of the differing resistance terms can be seen in Figure 1.1.

Antimicrobial agent (antimicrobial category)	**Isolate no 1 (PDR)**	**Isolate no 2 (XRD)**	**Isolate no 3 (MDR)**
gentamycin (aminoglycoside)	X	X	
tobramycin (aminoglycoside)	X		
amikacin (aminoglycoside)	X		
netilmicin (aminoglycoside)	X		
imipenem (carbapenem)	X	X	X
meropenem (carbapenem)	X	X	
doripenem (carbapenem)	X	X	
ceftazidime (cephalosporins)	X		X
cefepime (cephalosporin)	X	X	
ciprofloxacin (fluoroquinolones)	X	X	X
levofloxacin (fluoroquinolones)	X		
piperacillin-tazobactam (penicillin + b-lactamase inhibitors)	X		
ticarcillin-clavulanic acid (penicillin + b-lactamase inhibitors)	X	X	
aztreonam (monobactam)	X	X	
fosfomycin (phosphonic acid)	X		
colistin (polymyxin)	X		
polymyxin B (polymyxin)	X		

Antimicrobial resistance presents several important consequences, affecting both the medical and agricultural sectors. Rising resistance levels of a variety of bacteria increases the likelihood of infections becoming untreatable, whilst simultaneously increasing mortality rates, especially among immunocompromised patients (Bhatt *et al.,* 2014). As well as this, overall expenditure required for treatment, is increased, as alternative, more expensive last resort drugs are used (Bhatt *et al.,* 2014; Gebreyohannes *et al.,* 2013). Additionally, increased hospital stays due

to prolonged infection, and potential lengthened monitoring of patients, due to increased toxicity of treatment/antibiotics to overcome this infection, further increases the overall expenditure (Laxminarayan *et al.,* 2013; Santajit and Indrawattana, 2016). A recent review by O' Neill (2016) highlighted the dire consequences of AMR, including the effect it will have on the ability to perform simple surgical procedures, such as caesarean sections and joint replacements, due to an increased likelihood of resistant post-operative infections and hence an increased likelihood of mortality (Bhatt *et al.,* 2014; Bala Chandrasekhar *et al.,* 2015; Mangram *et al.,* 1999).

Certain bacterial species, known as the ESKAPE pathogens, have also been highlighted as a key risk, calling for the urgent development of new antimicrobials (Rice, 2008). This acronym includes the bacteria *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pnuemoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and the *Enterobacter* species, which are mainly associated with nosocomial infections and are increasingly seen to be resistant towards antimicrobials (Rice, 2008; Santajit and Indrawattana, 2016). The World Health Organisation (WHO), who have defined critically important antimicrobials for use in human medicine, also recently highlighted the urgency of this issue, and released a list, prioritising drug resistant organisms that require urgent research and development into new antibiotic treatments (WHO, 2016; WHO 2017). Carbapenem resistant *A. baumannii*, *P. aeruginosa* and *Enterobacteriaceae*, that also have the ability to produce Extended Spectrum Beta Lactamases (ESBLs), are detailed as a critical priority, and are top of the list of organisms that require desperate research into novel antimicrobials against them (WHO, 2017).

1.2 Overcoming this antibiotic resistance

WHO (2016) have provided several objectives for a global action plan against AMR, working to raise awareness of this resistance and its effects on the health system, as well as on the prevention of infections and unnecessary use of antimicrobials in both human and veterinary medicine. In a final report tackling AMR, O'Neill (2016) also highlighted 10 means of reducing the demand of antimicrobials, including increasing global awareness as well as surveillance of drug consumption, and resistance, among others. In addition, there remains a great need for the research and development of novel antimicrobials, necessary in the fight against AMR, which could include the chemical manipulation of antibiotics currently in use, as well as seeking new products from natural sources (Shore and Coukell, 2016; The PEW Charitable Trust, 2016).

Chemical modification of already known antibiotics has proven useful in providing further antibiotics, but completely new classes of these drugs are needed to overcome resistance (Zinner, 2005). This is, however, a major challenge, due to the current 'discovery void', with a severe lack of novel antimicrobials reaching the market. No new major antibiotic classes were discovered between 1987 and 2015, with teixobactin, an antibiotic with action against Gram-positive bacteria,

being the first new class in over 30 years (Silver, 2011; Ling *et al.,* 2015). There have only been two new antimicrobial classes developed and released for use from the pharmaceutical market since 1985, linezolid, an oxazolidinone, in 2000 and daptomycin, a lipopeptide, in 2003, however these classes were reported much earlier in 1978 and 1987 respectively, demonstrating just how slow the development of novel antimicrobials has been in recent years (Butler and Buss, 2006; Silver, 2011). This lack of research is mainly due to the large cost of research and development of new antimicrobials, which outweighs the money returned from these drugs. Courses of these antibiotics are often short (days), compared to drugs used for treatment of chronic conditions such as diabetes, hence research for chronic conditions is favoured by pharmaceutical companies (Zinner, 2005; O'Neill 2016).

Isolation of natural resources plays a significant role in the discovery of new drugs and have been essential for innovation and production of compounds for use in clinical settings (Gullo *et al.,* 2006). Between the years 1981 and 2002, over half the antibacterial drugs approved for use were developed from natural compounds (Newman *et al.,* 2003). An example of the significance of natural products is the antibiotic mupirocin, produced by the soil bacterium *Pseudomonas fluorescens*, which was initially isolated in 1971 and introduced into a clinical setting in 1985 (Newman *et al.,* 2003; Sutherland *et al.,* 1985). The majority of antimicrobials that have been synthesized and are commonly seen today in medical practices, originated from secondary metabolites produced by soil microorganisms, with the antibiotic mupirocin being one example (Kavitha *et al.,* 2010).

Teixobactin, mentioned above, is produced by the Gram-negative bacterium *Eleftheria terrae,* which was recently isolated in 2015 from soil, using an iChip device (Ling *et al.,* 2015). This device allowed bacterial organisms to be cultivated in their natural environment, enabling the growth of more bacteria that are unculturable under laboratory conditions (Ling *et al.,* 2015). This novel antimicrobial, whose mechanism of action targets bacterial cell wall synthesis, was shown to have good activity against Gram-positive organisms, with inhibitory effects even being seen against vancomycin-resistant Enterococci. This discovery highlights soil as a valuable source of novel secondary metabolites, that could potentially help overcome AMR (Ling *et al.,* 2015). Teixobactin is an example of a new antimicrobial reaching the market, however due to its ineffectiveness against Gram-negative organisms, such as those belonging to the *Enterobacteriaceae* family, it further emphasizes the need for additional research and development into novel antimicrobials. Additional new antimicrobials are needed in this fight against AMR, as the overuse of this one new antibiotic will again lead to development of resistance towards it (Ling *et al.,* 2015).

The search for new antimicrobials is therefore of significant importance. As such, searching out potentially inhibitory compounds from new and unexploited settings is a possible area of

interest in the early stages of drug discovery. The current study focusses on returning to more natural resources, in the hope of locating antimicrobial producing organisms (APO), and hence begin to remedy the major threat affecting both the pharmaceutical industry and the medical sectors, that is, the increasing amount of resistance being seen by varied species of bacteria.

1.3 Actinomycetes and *Streptomyces* spp.

Actinomycetes, a class of Gram-positive filamentous bacteria, are often located in soil and marine environments. They are known to be great producers of natural secondary metabolites, including antimicrobials, of which several are currently in use today (Gebreyohannes *et al.,* 2013; Kavitha *et al.,* 2010). Several of the bioactive compounds produced by genera in the Actinomycetes group have been seen to act on organisms such as methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant Enterococci (VRE), among others, showing further potential of Actinomycetes for the production of compounds able to overcome the bacterial resistance currently being seen (McArthur *et al.,* 2008). More than 70% of all known antibiotics originated from soil microorganisms from the Actinomycetes group, with the majority of these being produced from the *Streptomyces* genus (Sanglier *et al.,* 1996; Ganesan *et al.,* 2016).

The *Streptomyces* spp. have been shown to be a reliable producer of active metabolites, with around 75% of antibiotics produced by organisms within the Actinomycetes group alone, and currently in commercial use, derived from this *Streptomyces* genus (Sharma *et al.,* 2016; Miao and Davies, 2010). Table 1.2 displays several antimicrobial compounds that have been isolated from the *Streptomyces* spp., among other bacteria within the Actinobacteria class (Miao and Davies, 2010).

Due to geographical variation in the overall ecosystems and microorganisms present in soil, it is likely the presence of antibiotic-producing Actinomycetes within this microbiome is also variable, and hence returning to the study and isolation of Actinomycetes and other microorganisms in supposed unexplored soil ecosystems, allows the potential for novel inhibitory compounds to be found (Singh *et al.,* 2012).

Table 1.2: Commercial therapeutic drugs, originally produced by organisms belonging to the Actinobacteria class (adapted from Miao and Davies, 2010).

Compound	Medical application	Antimicrobial-Producing organism
tetracycline	Antibacterial	*Streptomyces aureofaciens*
streptomycin	Antibacterial	*Streptomyces griseus*
kanamycin	Antibacterial	*Streptomyces kanamyceticus*
cefoxitin	Antibacterial	*Streptomyces lactamdurans*
vancomycin	Antibacterial	*Amycolatopsis orientalis*
chloramphenicol	Antibacterial	*Streptomyces venezuelae*
ivermectin	Antihelminthic	*Streptomyces avermitilis*
nystatin	Antifungal	*Streptomyces noursei*
bleomycin	Anticancer	*Streptomyces verticillus*

A study by Iliĉ and colleagues (2007), characterised the antibacterial, as well as antiviral activity of secondary metabolites produced specifically by *Streptomyces* spp. isolated from soil from southeast Serbia. A total of 20 different Actinomycetes were isolated, with the five most active isolates being identified as *Streptomyces hygroscopicus,* which were seen to have antimicrobial activity against all the test organisms used; this included *P. aeruginosa, Enterobacter aerogenes*, *Candida albicans*, as well as anti-viral activity against the Herpes Simplex virus. A more recent study by Ganesan and colleagues (2016), aimed to locate Actinomycetes in soil samples from Tamil Nadu, India, and determine inhibitory activity towards fungi, as well as bacteria. Forty-four out of a total of 106 Actinomycetes strains isolated, displayed antimicrobial activity against at least one of the different pathogenic microbes, with five of these showing broad inhibitory activity against the majority of the 24 microbes used, including inhibitory action against *Proteus vulgaris, Klebsiella pneumoniae* and the fungi *Candida tropicalis*. One of these five highly inhibitory strains also showed inhibition towards all 24 of the test organisms used, which was identified to have 98% similarity to *Streptomyces rimosus*, using DNA sequencing results. This study, combined with the findings by Ilic and colleagues (2007), display that novel compounds with a broad range of inhibitory activity are still being produced and isolated from the *Streptomyces* genus, and that the Actinomycetes group have immense potential in the search for new antimicrobial compounds.

Further studies have also isolated extracellular metabolites from Actinomycetes, focusing on those with antifungal potential (Sharma and Parihar, 2010). Kavitha and colleagues (2010) isolated four different Actinomycete strains, three of which were from the *Streptomyces* genus, with one of these producing metabolites with strong antifungal activity against such fungi as *Aspergillus flavus* and *Aspergillus niger*. Microorganisms from the *Streptomyces* genus only, have

also been seen to produce compounds with antifungal activity. Twenty-seven (6.8%) out of the 396 *Streptomyces* isolates obtained from soil samples, collected from the rhizosphere of 16 medicinal plants in Thailand by Khamna and colleagues (2009), were seen to produce antifungal activity towards at least one of the six pathogenic fungi tested. One *Streptomyces* strain obtained, was seen to strongly inhibit all the test fungi. This study focussed on inhibitory activity towards fungi that were pathogenic towards plants, which demonstrates the need for inhibitory compounds to work against crop pathogens in the agricultural industry, as well as the research needed for the production of novel antimicrobials for use in the medical sector, to overcome the ever-increasing resistance being seen.

1.4 Factors affecting antimicrobial compound production

As well as sampling areas of soil alone, areas where animals and livestock influence the soil microbiome may affect the presence of organisms with the ability to produce inhibitory compounds. Farmlands could be especially resource-rich in the hunt for novel antimicrobials, due to the effect the local environment and farm animals may have on the ecosystem, as well as the increased likelihood of antimicrobial use, that is likely to affect the resulting microorganisms in the samples (Hyde *et al.,* 2017). Few of the studies on the isolation of soil microorganisms have focussed on the isolation of organisms from areas where significant numbers of livestock reside. One study by Sharma and colleagues *(*2016) investigated the antimicrobial potential of a *Nocardia* strain they isolated from a wildlife sanctuary in India, finding that it had strong inhibitory activity against Gram-positive and Gram-negative bacteria, as well as yeasts. Bizuye and colleagues (2013) also investigated the isolation of Actinomycetes for the production of antibiotics in soils, in North West Ethiopia, with a cattle breeding area being one of the five sites from where soil was sampled. Ten out of 30 Actinomycetes isolated, originated from this breeding area, with two of these displaying antimicrobial activity against one of the test bacteria and fungi used. Upon further screening it was determined that both the isolated Actinomycetes from the breeding ground had activity against both Gram-positive and Gram-negative bacteria, with one of them having further activity against fungi (Bizuye *et al.,* 2013). These studies may highlight that the collection of samples where the soil microbiome is affected by the presence of animals, is a promising area for the location of antimicrobial producing Actinomycetes, and other organisms of interest.

The isolation of Actinomycetes/*Streptomyces* spp. from soil and natural environments can be seen to still be a viable method for the isolation of inhibitory compounds with a vast range of activity towards differing organisms. However, the mechanisms behind which antibiotics are produced by these organisms are still not completely understood, but it is known that there are several intermediate stages and molecules/metabolites involved, which are influenced by the external environment conditions, as well as several methods of regulation including precursor

accumulation, transport of nutrients and regulation of gene expression (Rokem *et al.,* 2007). It has been noted that adverse or stressful growth conditions encourage microorganisms such as *Streptomyces* spp. to produce antibiotics. Hayes and colleagues (1997) showed that *Streptomyces coelicolor* produced methylenomycin, an antibiotic, under alanine-limited conditions, and/or a triggered acidic pH shock, through increased transcription of the *mmy* gene and hence increased production of methylenomycin. However, heat and alkali pH shock, as well as the addition of alcohol had no effect on triggering the production of methylenomycin, and hence it was shown that the antibiotic production occurs as a specific stress response to particular conditions, not any general stress conditions (Hayes *et al.,* 1997). This displays that environmental factors, such as nutritional availability and pH, play a major role in the production of antibiotics.

The need for discovery and development of new antimicrobials is a growing concern in both the world of human and veterinary medicine. This study aims to continue this search for novel secondary metabolites by using forestry and farmland sampling for the isolation of APO, and *Streptomyces* spp. using active selection. As far as it is known Friston Forest, New Forest, and the farmland areas sampled are unexplored, and hence could lead to the isolation of potential APO.

1.5 Hypothesis

It has been hypothesized that the untested forestry and farmland areas selected, contains soil microorganisms with the ability to produce novel inhibitory compounds, able to inhibit the growth of pathogenic strains of bacteria, including both Gram-negative and Gram-positive organisms. Within these isolated organisms, designated as antimicrobial producing organisms, a large quantity is theorised to belong to the *Streptomyces* genus, a common soil bacterium with a known ability to produce a vast amount of secondary metabolites.

The isolation of pathogenic bacteria, from the same soil samples collected for the isolation of the microorganisms with inhibitory ability, has also been hypothesized, due to the varying microbiomes. As far as is known, these forestry and farmland areas have not yet been tested for the presence of microorganisms, both pathogenic as well as those presenting antimicrobial activities, and hence it is hypothesised that isolation of novel compounds is more likely.

1.6 Aims

The present study aims to use the soil samples collected from Friston and New forest, as well as Dawes Farm, to isolate and determine the prevalence of organisms with the ability to produce compounds, able to inhibit the growth of selected test bacteria. Using sensitive and resistant test bacteria within the perpendicular streak method, as well as a newly developed

starvation method, the extent of the inhibitory activity of these compounds and their producers can be determined.

Using the same soil samples, this study also aimed to isolate and determine the prevalence of selected pathogenic bacteria in the soil samples collected, using a range of biochemical tests to identify organisms isolated to a genus level, and allow comparative analysis to organisms with inhibitory ability isolated from the same locations.

Using determinative PCR (polymerase chain reaction), and primers specific to the 16S rRNA gene of the *Streptomyces* spp., this study's final aim is to determine the extent of organisms belonging to this genus, with all isolated bacteria, from the 3 locations, presenting morphological resemblance to this genus, being tested.

2.0 Materials and Methods

2.1 Materials

All broth and agar media were autoclaved at 121°C at a pressure of 15 psi for 15 minutes for sterilisation.

2.1.1 Media

Several agar and broth media were prepared using the instructions provided. These included Nutrient agar (Oxoid, CM0003), Mannitol Salt Agar (Oxoid, CM0085), Edwards modified medium (Oxoid, CM0027), Simmons Citrate agar (Oxoid, CM0155), and MacConkey, both with and without crystal violet (Sigma, M8302, and Oxoid, CM0007 respectively), Mueller-Hinton agar (Oxoid, CM0337), Nutrient broth (Oxoid, CM0001), and Tryptone soy broth (Oxoid, CM1016).

2.1.1.1 Oat agar

Oat agar was used for the cultivation and isolation of APO, specifically *Streptomyces* spp. (Shepherd *et al*., 2010). Fine oats (60 g) were added to 1 L of distilled water and left to steep for 30 minutes, before passing through a cheesecloth to remove solid matter. Bacteriological agar (12.5 g) (Oxoid, LP0011) was added to the oat mixture before the volume was made up to 1 L using distilled water, before autoclaving as above in section 2.1.

2.1.1.2 Potato starch agar

Potato starch agar was used for the cultivation and isolation of APO, specifically *Streptomyces* spp. Potato starch (10 g) (Fisher Sci, 15413047), gelatin peptone (5 g) (Oxoid, LP0008), beef extract (3 g) (Oxoid, LP0029) and bacteriological agar (15 g) (Oxoid, LP0011), was added to 1 L of water before autoclaving as described in section 2.1. Post-autoclaving 50 µg/ml anti-fungals, cyclohexamide (Sigma 01810) and nystatin (sigma N4014), was aseptically added to cooled oat and potato starch agar. Both potato starch and oat, as well as nutrient agar plates and slopes were used for storage of the APO, and all other isolates.

2.1.1.3 Mannitol soy agar

Mannitol soy agar was used for the cultivation and sporulation of potential *Streptomyces* spp. isolates. Soya flour (20 g), Mannitol (20 g), bacteriological agar (20 g) (Oxoid, LP0011) and Magnesium (5 mM) were added to 1 L of distilled water, before autoclaving as stated in section 2.1.

2.1.1.4 Plate count broth and agar

Plate count broth, was made using 5 g peptone (0.5%) (Sigma, 70169), 2.5 g yeast extract (0.25%) (Sigma, 92144) and 1 g of glucose (0.1%) (Sigma, G8270) in 1 L of distilled water. Plate count agar was made using the same concentrations of products with the addition of 15g of

bacteriological agar (1.5%) (Oxoid, LP0011) to this mixture. Both plate count broth and agar were autoclaved as described in section 2.1.

2.1.2 Biochemical characteristic testing products

Tryptone water was made for use in indole testing using 10 g of Tryptone (Sigma, T9410) and 5 g of sodium chloride (Sigma, S7653) in 1 L of distilled water, before being autoclaved as stated in section 2.1, and Kovacs reagent (SLS, CHE2280) was used according to instructions. Urea broth (Sigma, 51463) was made according to instructions, and sterilised using filtration. Ringers solution (Sigma, 96724) was made according to instructions for McFarland standardisation of bacteria; one tablet was dissolved in 500 ml of distilled water and autoclaved as stated in section 2.1. Other media including Phosphate Buffered Saline (PBS) (Thermofisher, 003002) and selective agar (see section 2.1.1) were made according to instructions and autoclaved as described in section 2.1.

Oxidase reagent was made by mixing 0.01 g of N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) (Sigma, 87890) in 1 ml of distilled water. Catalase reagent (2% Hydrogen peroxide (v/v)) was made by mixing 1 ml of 20% Hydrogen peroxide into 9ml of sterile distilled water. Analytical Profile Index (API) testing was carried out using the API 20E kit, following instructions for correct use (bioMérieux). Coagulase test was carried out according to instructions stated in the Staphylase test kit (Oxoid, DR0595).

2.1.3 Bacterial cultures

Test bacteria *Escherichia coli* (NCTC 11954), *Staphylococcus aureus* (NCTC 6571), *Staphylococcus epidermidis* (NCTC 13360), *Pseudomonas aeruginosa* (NCTC 13359), *Proteus mirabilis* (NCTC 13376), *Streptococcus pneumoniae* (NCTC 7465), and *Salmonella* Typhimurium (NCTC 10787) were obtained from Kingston University Culture Collection, London.

Clinical strains of test bacteria, stored at Kingston University, were isolated from differing locations. *P. mirabilis*, and *E. coli* were obtained from Dr Brian Jones at the University of Brighton, UK, and from Dr Jackie Kenny at the Royal Marsden hospital, Sutton, London, UK. NCTC control strain of Methicillin-Resistant *S. aureus* (MRSA) was also used (NCTC 12493).

Streptomyces coelicolor, strain M145, used as a positive control in PCR and gel electrophoresis for the identification of *Streptomyces* spp. was kindly provided by Dr Paul Hoskisson from the University of Strathclyde, UK. All isolates were stored at -80°C using cryovials.

2.1.4 Genomic DNA

DNA isolated from *Streptomyces coelicolor*, M145, also provided by Dr Paul Hoskisson at a final concentration of 13 ng/µl, was used as a positive control in the Polymerase Chain Reaction (PCR), and gel electrophoresis for the identification of *Streptomyces* spp. (see section 2.2.3.3).

2.1.5 Polymerase chain reaction and gel electrophoresis

Primers specifically targeting the 16S rRNA gene belonging to the *Streptomyces* spp. were provided from Dr Paul Hoskisson from the University of Strathclyde (Kirby *et al.,* 2011) (Table 2.1). Tris-Borate-EDTA (TBE) buffer was made prior to PCR for use as gel material, and for the running of the gel, using 10.8 g Tris base, 5.5 g Boric acid, and 0.75 g Ethylenediaminetetraacetic acid in 1 L of distilled water, at a final pH of 8.0.

Table 2.1: Forward and reverse primers used for the amplification of the 16S rRNA gene, for the detection of *Streptomyces* spp. designed by Dr. Paul Hoskisson (Kirby *et al.,* 2011).

Forward	Reverse
27S (5' – AGAGTTTGATCCTGGCTCAG – 3')	1492R (5' – TACGGCTACCTTGTTACGACTT – 3')

2.2 Methods

2.2.1 Sample collection

Samples were aseptically collected to fill sterile 50 ml falcon tubes using gloves, at several areas in duplicate within the Friston Forest (Modern Forest) and New Forest (Ancient Forest) areas, with soil disturbed using a clean trowel and collected using the falcon itself. Samples were also taken at several sites at Dawes Farm, (Warnham, UK) using the same method, with geographical coordinates (longitude and latitude), and area information noted down at location (see appendix tables A1, A3 and A8). Forty-two samples were collected from New Forest (Hampshire, UK), in November 2016. Twenty samples were taken from Friston Forest (East Sussex, UK), and 22 samples were taken from Westpoint Farm Vets, Dawes Farm, in April 2017 and May 2017 respectively. All samples were labelled and transported to the lab for further testing, and stored at 4°C.

2.2.2 Isolation of microorganisms from the soil samples

2.2.2.1 Optimisation of the isolation of microorganisms from the soil samples

Initially, soil samples were directly streaked onto all agars used: MSA; Nutrient; MacConkey; and Edwards agar. However, to make isolation less complex, and prevent the specificity of the different selective agar having an effect on the organisms isolated, soil samples were initially plated onto nutrient agar and further cultured onto selective agar, according to Gram stain. Both 1/10 and 1/100 dilutions of soil diluted in Ringer solution were tested, with a 1/100 dilution being deemed as optimal, due to the denser cultures a 1/10 dilution presented.

2.2.2.2 Isolation of pathogenic organisms

Previously selected pathogenic strains were targeted to be isolated from soil samples if present: *E. coli; Salmonella* spp.; *Klebsiella* spp.; and *Streptococcus faecalis* were highlighted as important in the isolation of bacteria from all forest and farmland soil samples.

A 1/100 dilution of the soil samples was made by adding 0.25 g of soil to 24.75 ml of sterilised Ringers solution and vortexed to resuspend the soil in the solution. Samples were spread over nutrient agar, using a sterile cotton swab, before being incubated for 24 hours at 37°C. Bacterial isolates were then subcultured onto one half of nutrient agar plates, according to differences in morphology to achieve pure cultures on nutrient agar, which was confirmed via Gram stain.

2.2.2.3 Isolation of Antimicrobial Producing Organisms and *Streptomyces* spp.

Five grams of each soil sample was placed into empty sterilised petri dishes and dried for 5-7 days in the incubator at 45°C. The aim of this was to reduce the levels of Gram-negative bacteria in the sample to aid the isolation for *Streptomyces* spp. (Chaudhary *et al.,* 2013).

Dried soil samples were diluted to 1/100 and spread on agar using the same method as above (see section 2.2.2.2). The farm and Friston Forest samples were streaked onto both potato starch and oat agar plates. New Forest samples were streaked onto potato starch agar only, due to the further optimisation of agar, and development of methodology after the New forest samples were initially tested. All plates were incubated for 7-10 days at 25°C for optimal growth of the *Streptomyces* spp. Bacteria presenting areas of growth inhibition towards other organisms, grown from the same soil sample, and/or similar colony morphology to that of *Streptomyces* spp. were selected and sub-cultured onto nutrient agar until pure cultures were obtained. All APO were numbered according to the total amount obtained from the three locations in total. Pure samples were maintained using the cultivation agar of choice (potato starch or oat agar). Samples were also stored using cryovials and nutrient agar slopes at -80°C and room temperature respectively.

2.2.3 Identification and characterisation of isolated bacteria

2.2.3.1 Optimisation of the identification and characterisation of isolated bacteria

PCR was optimised for the identification of *Streptomyces* spp. from the APO isolated using the amplification of the 16S rRNA gene from this genus. *Streptomyces coelicolor* and its previously extracted DNA, provided by Dr Paul Hoskisson, was used to allow the optimisation of the reaction mixture, before performing the protocol on DNA extracted from APO. Varying concentrations of both DNA and primers were tested; 4 ng/µl, 2 ng/µl, 1 ng/µl, and 0.5 ng/µl, concentrations of DNA were tested, as well at the use of 1 µM/µl, 500 nmol/µl, 100 nmol/µl, 10 nmol/µl, 1 nmol/ µl, and 100 pmol/µl, concentrations of primer in the final reaction mixtures. Test DNA was chosen to be standardised at ~2 ng/µl, with a primer concentration of 1 µM/µl used in the final reaction mixtures. Both final concentrations of 5% and 0.2% (v/v) DMSO we also tested in the final 25 µl reaction mixture, with 5% being optimal. Both a 1% as well as a 2% (w/v) agarose gel were tested for optimal separation of the DNA, with a 2% gel detailing better band separation.

2.2.3.2 Identification and characterisation of pathogenic bacteria of interest

'Pure' cultures of different bacterial isolates collected from the soil were subjected to various biochemical tests, to allow further identification of the bacterial species present (Table 2.2). Initial Gram staining allowed identification of the shape and Gram reaction of the individual isolates, as well as confirming cultures were pure. Using a sterile metal loop, the bacterial samples were added to 10µl of distilled water already placed on a slide, with the cells then being fixed using a Bunsen flame. Stains were added accordingly to samples using the minute method; in order Crystal violet, Gram's iodine, alcohol solution (80% methanol and 20% acetone (v/v)), and Carbol fushin were added, with distilled water used to rinse slides between stains (Yazdankhah *et al.,* 2001). It should be noted that the alcohol solution however was left on slides for 30 seconds only. Slides were then dried using paper towel and were viewed under the microscope at x1000 oil immersion magnification. Apparent mixed cultures were further streaked, and again Gram stained. Cell shape, Gram stain, and colony morphology was noted alongside isolate numbers.

Isolates were plated onto selective media according to their Gram stain. Gram-positives were plated on Mannitol salt agar, and Edwards modified medium for the detection of the *Staphylococcus* spp. and *Streptococcus* spp. respectively (Abiola and Oyetayo, 2016). Gram-negative bacteria were streaked onto MacConkey agar, both with and without crystal violet, to confirm the presence of Gram-negative bacteria; the inclusion of crystal violet renders the medium more selective for Gram-negative bacilli (See section 2.1.1) (Abiola and Oyetayo, 2016). Biochemical tests were used to identify the isolated pathogens from the soil. Results from catalase, oxidase, citrate, indole, urea, and API testing were used to identify isolates collected from the non-dried

soils samples, to a species level (see sections 2.1.1 and 2.1.2) (Cowan and Steel, 2003; Abiola and Oyetayo, 2016). Positive and negative controls were used in each individual biochemical test to confirm the accuracy of results, according to table 2.2, using the test organisms stated in section 2.1.3.

Table 2.2: The several biochemical tests used for the identification of organisms isolated from the wet soil samples. The test organisms used as positive and negative controls for the individual tests are given, with the test strains used stated in section 2.1.3 used (Abiola and Oyetayo, 2016). The individual biochemical testing products can be seen in section 2.1.2.

Biochemical test	**Biochemical test analysis**	**Positive biochemical test result**	**Test organisms used to confirm positive (+VE) and negative (-VE) results for biochemical test.**	
			+VE	**-VE**
Gram Stain	Determination of the cell wall structure and shape (Gram-positive or Gram-negative bacterium).	Purple stain retained by the cell wall – visualised by microscope.	*Staphylococcus* spp.	*Enterobactericeae* (e.g *E. coli*, *Salmonella* spp. etc)
Catalase test	Determination if the bacterial isolate contains the catalase enzyme.	Bubbles are seen upon the addition of the bacteria to hydrogen peroxide.	*Staphylococcus* spp.	*Streptococcus pneumoniae*
Oxidase test	Positive result indicates the presence of the cytochrome C oxidase enzyme (indicates the bacteria is aerobic).	The appearance of a blue colour after the addition of bacteria to filter paper coated with TMPD.	*Pseudomonas aeruginosa*	*Escherichia coli*
MacConkey agar	Growth of Gram-negative bacteria only. Determination of Lactose fermentation.	Positive lactose fermentation result is indicated by growth of pink colonies.	*Escherichia coli*	*Proteus mirabilis*
Simmon's citrate test	Positive result for organisms with the ability to use citrate as a carbon source.	Visible bacterial growth and/or a colour change of the agar to blue.	*Salmonella* Typhimurium	*Escherichia coli*
Urease test	Positive result indicates the presence of the urease enzyme and ability to utilise urea.	Presence of the urease enzyme indicated by the colour change of the media to pink.	*Proteus mirabilis*	*Escherichia coli*
Coagulase test	Differentiation of *Staphylococcus* spp. using the ability of the organism to convert fibrinogen to fibrin.	Agglutination of the plasma added indicates a positive result.	*Staphylococcus aureus*	*Staphylococcus epidermidis*
Indole test	Ability of the organism to convert tryptophan to Indole.	A pink colouring of the liquid is produced.	*Escherichia coli*	*Staphylococcus aureus.*
Mannitol Salt agar	Selective for Gram-positive bacteria. Distinguish between *Staphylococcus* spp. using mannitol fermentation.	Colour change of pink/red agar to yellow indicates a positive result for the ability to ferment mannitol.	*Staphylococcus aureus.*	*Staphylococcus epidermidis.*
Edwards modified medium.	Specific for the isolation of *Streptococcus* spp.	Growth on agar indicates a positive result for the presence of *Streptococci* spp.	*Streptococcus pnuemoniae.*	*Escherichia coli*

2.2.3.3 Identification and characterisation of Antimicrobial Producing Organisms and *Streptomyces* spp.

Gram staining was again used for confirmation of pure cultures, of the organisms isolated from the dried soil samples, selected due to seen inhibition and/or similar colony morphology to that of *Streptomyces* spp. Actinomycetes (e.g. *Streptomyces* spp.) were selectively identified according to its known long rod shape using the Gram stain method, as well as its colony morphology; on nutrient agar they often present thick, hard to move colonies, with pigmentation often seen. Different aerial and substrate mycelium colours were also used to selectively isolate those potentially belonging to the *Streptomyces* genus.

The identification of *Streptomyces* spp., was confirmed using Polymerase Chain Reaction (PCR) and primers specific for the 16S rRNA gene, found within *Streptomyces* spp. (Table 2.1).

Prior to the PCR protocol, a sterile loop was used to inoculate the potential *Streptomyces* spp. onto mannitol soy agar before incubation for 5-7 days at 25°C, allowing time for sporulation. The agar plates were then flooded using 20 ml of 20% (v/v) glycerol solution, (diluted using distilled water before autoclaving as above), and a cotton bud was used to swab the agar surface to dispense the potential *Streptomyces* spores into solution. Two millilitres of this glycerol solution was then placed into 100 ml of Tryptone soy broth before incubating with shaking at 25°C for ~ 2 days ready for DNA extraction. One millilitre of each sample in tryptone soy broth was then placed into sterile Eppendorf tubes in duplicate and centrifuged at 10,000 x g for 5 minutes. The supernatant was discarded before resuspension in 1 ml of PBS, by vortexing. Centrifugation and resuspension was again repeated as above in PBS, before placing Eppendorf's in a heat block at 105°C for 10 minutes. Eppendorf tubes were then centrifuged again for 5 mins at 10,000 x g, before measuring the DNA concentration and purity of the supernatant in triplicate using the Nanovue spectrophotometer. The mean DNA results were recorded for each of the isolates tested from the 3 locations. The supernatant was further diluted 1/10 (v/v) using PBS, in preparation for PCR.

The DNA extracted as above, was added alongside correct volumes of the primers, water, Dimethylsulfoxide (DMSO) (Sigma, D2650) and DreamTaq (Fisher Scientific, 11813933) (Table 2.3), into a sterile 0.2 ml Eppendorf tube before being placed into a thermal cycler. A no DNA negative control was used, with the DNA volume of the reaction mixtures replaced with sterilised water. Table 2.4 details the PCR conditions, provided by Dr Paul Hoskisson, for amplification of the 16S rRNA gene in *Streptomyces* spp. Five microliters of each sample or 100bp plus DNA ladder (Thermofisher, SM0321) were added to each well in a 2% agarose gel (see below) and run at a voltage of 65V (PowerPac^{TM} Biorad) for ~105 minutes. DNA band visualisation was then achieved using the Gbox, with clear bands at ~1500bp indicating organisms belonging to the *Streptomyces* genus, ensuring no band was present in the no template DNA well.

For the separation of DNA, a 2% (w/v) agarose gel was made by mixing 1.50 g of agarose in 75 ml of TBE buffer (see section 2.1.5), and heating until a homogenous clear liquid state is reached. After slight cooling, nine microliters of SYBR safe (Fisher Scientific 10328162) was added to the gel before pouring into cassette and placing in the well comb, leaving the gel to set. Once set, combs were removed, and gels were placed into a horizontal Sub-Cell electrophoresis cell (Biorad) before being immersed in ~250 ml TBE buffer, ensuring the gel was well covered.

Table 2.3: The reaction mixture used in PCR for the detection of *Streptomyces* spp.

Component	Volume (µl)
Dream Taq	12.5
Forward primer	1.00
Backwards primer	1.00
Template DNA	1.00
Nuclease free water	8.25
100% DMSO (5% final concentration)	1.25
Total volume (µl)	**25**

Table 2.4: The PCR conditions used for the amplification of the 16S rRNA gene within the *Streptomyces* spp.

Step	Temperature (°C)	Time (mins)	No. of Cycles
Initial Denaturation	95	10	1
Denaturation	95	1	30
Annealing	50	0.75	
Extension	72	1.5	
Final Extension	72	10	1

2.2.4 Further testing of Antimicrobial Producing Organisms

Inhibitory activity exerted by the APO isolated from Friston Forest, New Forest, and Dawes Farm samples, was established using several methods against a range of both sensitive laboratory strains and clinical isolates of test bacteria.

2.2.4.1 Optimisation of novel antimicrobial screening (perpendicular streak method)

Initially, potential APO were incubated for 0, 2, 5 and 7 days, before the addition of the test bacteria to the Mueller-Hinton plates, used for perpendicular screening, However, due to time constraints, incubation of the potentially inhibitory bacteria for 5 and 7 days prior, was determined as unnecessary and was not continued in the final experiment.

2.2.4.2 Novel antimicrobial screening against sensitive strains of test bacteria (perpendicular streak method)

Perpendicular streak method was used to determine antagonistic activity of the APO against sensitive strains of both Gram-negative (*E. coli, P. aeruginosa* and *P. mirabilis*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria (Singh *et al.,*2016; Gebreyohannes *et al.,* 2013). Both test bacteria and APO of interest were diluted to 0.5 McFarland (1.5 x 10^8 CFU/ml) in Ringer's solution, vortexing to mix (see section 2.1.3).

Diluted test bacteria were added, in duplicate, to diluted APO of interest, that had no prior incubation and 2 days previous incubation, at both 25°C and 37°C on Mueller-Hinton agar, at a 90° angle, (perpendicular streak), before further incubation at original growth temperature (Singh *et al.,*2016; Gebreyohannes *et al.,* 2013). Figure 2.1 displays the layout of the agar plates used to test APO in the perpendicular screening method. The APO were incubated with the test bacteria for up to 7 days, with routine checks for any inhibition occurring every other day. Inhibition by the APO against any of the test bacteria was recorded, and repeat experiments were carried out using the same APO for confirmation of inhibitory action. Isolates displaying inhibition against sensitive strains of bacteria were further tested using clinical isolates, including some that conferred resistance to differing antibiotics.

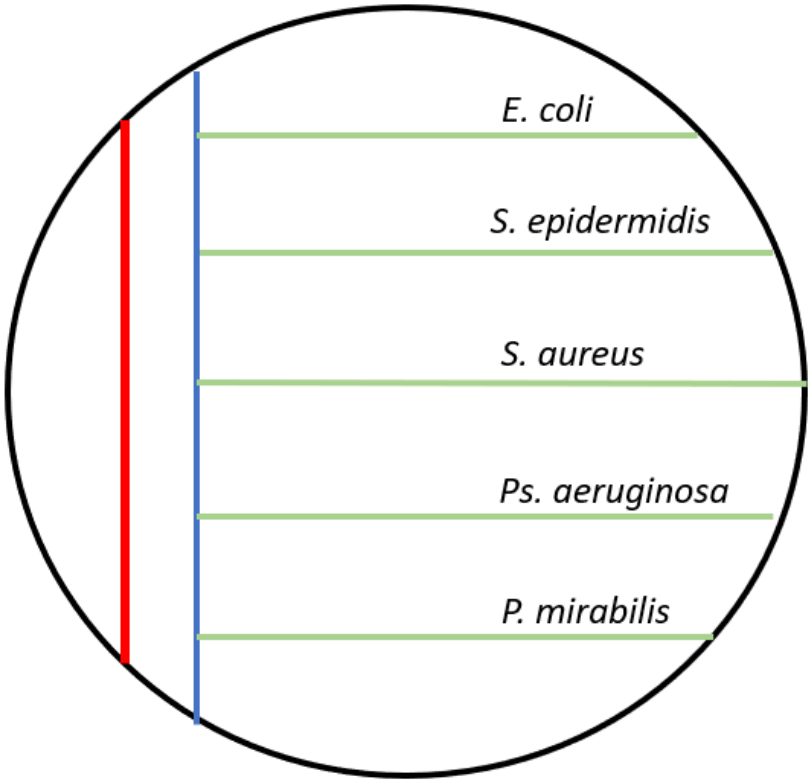

Figure 2.1: Displays an example of the layout of the Mueller-Hinton agar plates used to test the inhibitory activity of the APO against sensitive strains of test bacteria in novel antimicrobial screening. The red line indicates where the APO being tested was streaked, with the blue line on the right displaying the starting point at which the sensitive test organisms were streaked from towards the edge of the agar plate, causing the heaviest inoculum to be closer to where the APO was streaked.

2.2.4.3 Antimicrobial screening against clinical strains of test bacteria (perpendicular streak method)

Clinical strains of *Proteus* spp., *E. coli* and MRSA, were used to further test the inhibitory activity of APO of interest (see section 2.1.3). Prior to testing antibiograms were initially run to determine the sensitivity of the clinical strains used. Clinical strains of bacteria were diluted to 0.5 McFarland standard (1.5 x 10^8 CFU/ml), before being streaked as a lawn onto plates of Mueller-Hinton agar of ~4 mm in thickness, by swabbing in three directions across the plate. Selected antibiotic discs (see below) were then placed onto the surface of the plate using an antibiotic dispenser before being incubated at 37°C for 24 hours. Zones of inhibition were then recorded, and sensitivity of the strains was determined. *P. mirabilis* and *E. coli* were tested against amoxicillin and clavulanic acid (30μg), imipenem (10 μg), cefotaxime (5 μg), ciprofloxacin (5 μg) and gentamycin (10 μg). MRSA was tested using erythromycin (5 μg), oxytetracycline (30 μg), gentamycin (10 μg), ciprofloxacin (5 μg), and cefoxitin (30 μg) discs. Susceptibility was determined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint tables from 2017 (EUCAST,

2017). The data for oxytetracycline however was not available using this table, and hence results were reported for reference purposes.

Clinical strains of test bacteria were then exposed to APO of interest, in accordance to prior inhibition seen against specific test bacteria, using the same method as above (see section 2.2.4.2).

2.2.4.4 Optimisation of the starvation method

In pilot experiments centrifugation of the APO cultures in plate count broth occurred at 8000 x g for 5 minutes, however after transfer to plates large amounts of bacterial growth produced by the APO was seen, displaying a need for higher speed centrifugation to ensure more of the cells are pelleted.

2.2.4.5 Starvation method

All APO were further tested using the starvation method after initial screening above. APO were inoculated in the minimal medium of plate count broth (see section 2.1.1.4), for 7, 10 and 14 days in duplicate, at both 25°C and 37°C. One day prior to the end of the incubation of the APO of interest, sensitive strains of test bacteria (*E. coli, S. epidermidis*, and *S. aureus*) (section 2.1.3), were diluted in Ringer solution to 0.5 McFarland standard (1.5×10^8 cfu/ml) and streaked as a lawn using a sterile cotton swab, on both Mueller-Hinton and plate count agar plates, and incubated for 24 hours at 37°C. Using this same method *P. mirabilis* was also prepared for use in the starvation method, however plates were streaked only for APO that had previously displayed inhibition towards this organism (Friston Forest and Farm APO only). *P. aeruginosa* was not used in this method due to lack of inhibition seen towards this organism, and time constraints. After incubation, 1 ml of each organism was placed into an Eppendorf tube and centrifuged at 13,500 x g for 5 minutes to pellet the cells. Ten microliters of this supernatant was then added to plates containing lawns of test bacteria, in duplicate, and incubated for 7 days at both 25°C and 37°C. Figure 2.2 displays the layout of the agar plates used for further testing of the APO in the starvation method. Plates were monitored within this 7 day period and any inhibition caused by the supernatant was recorded. A further 2 ml of each broth inoculated with APO was centrifuged as above, allowing ~3 ml of each supernatant to be collected and stored at 4°C, for further use in testing. Repeats were carried out on isolate's that displayed inhibition against test bacteria on New Forest isolates only; for accuracy and reproducibility, the whole method was repeated, as well as a repeat using the stored supernatant collected from the initial round of experiments.

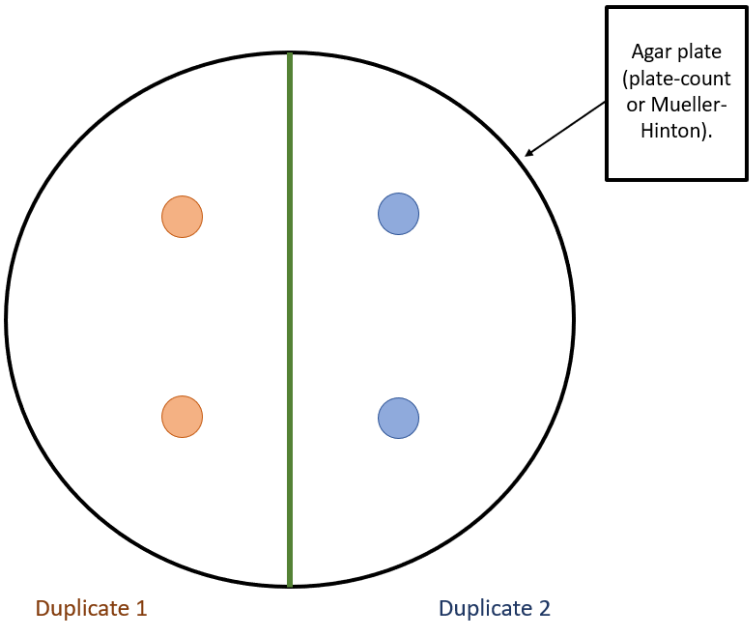

Figure 2.2: The layout of agar plates, either plate count agar, or Mueller-Hinton agar used to test inhibitory activity of APO supernatant in the starvation method. These plates were used to for standardised growth of the test bacteria (see section 2.2.4.5). A central line was drawn on the backs of the agar plates to distinguish between different broth cultures of the same APO which were grown in duplicate. Each duplicated growth of an APO was centrifuged and plated in duplicate again on one half of each agar plate.

2.2.5 Chemical analysis of potential novel antimicrobials- pilot study

The following methods were carried out in collaboration with Dr Adam LeGresley and Mr Rob Warren. Following the growth cycles reported in the starvation method (see section 2.2.4.5), the collected supernatant containing potential antibacterial components, was further analysed.

2.2.5.1 Solvent extraction

The supernatants, from the bacterial cultures of interest were harvested using centrifugation (5000 rpm) and using a 1:1 (v/v) volume of supernatant vs. solvent, liquid – liquid extraction was performed. To facilitate extraction a total of 3 solvents were used including petroleum ether, dichloromethane and acetonitrile. The samples were cleaned to remove impurities such as protein residue that may interfere with analysis, by acidification of the samples to pH2 using HCl (1 M) and incubated at 4^{o}C for 18 hrs. Following this incubation an equal volume of chilled acetone was added followed by filtration and neutralisation with NaOH (1 M). The residual acetone was removed by rotary evaporation before continuing with extraction.

The extraction was started using the least polar solvent, namely petroleum ether and stirred for two hours before performing the extraction. This was then followed by a dichloromethane extraction, shaking thoroughly, then finally an acetonitrile extraction. Due to

acetonitrile being miscible in water the salting out technique was used on this mixture (as described below in section 2.2.5.2). The individual fractions were then evaporated to dryness and NMR examination was carried out using a solvent of suitable polarity.

2.2.5.2 Salting out

The miscible layers in the solution were salted out using a 1:1 (v/v) volume of acetonitrile, and water and 1 M NaCl. This has shown to be a successful method for the extraction of erythromycin, having superior extraction efficacy and rapid phase separation compared to traditional liquid-liquid extraction (Le *et al.,* 2001).

2.2.5.3 Thin layer chromatography bioassay

The separated fractions from the solvent extraction (see section 2.2.5.1), were run on TLC plates for the separation of compounds, using optimised TLC solvent systems, that differed for each fraction and determined using trial and error (see results section 3.2.3.3). Spots were detected under ultraviolet light by spraying TLC plates with water and ninhydrin for the detection of hydrophilic compounds, and compounds containing free amino groups (Alajlani *et al.,* 2016). Each band was scraped off and extracted with MeOH 1M (methanol) to give a single compound for antibacterial testing and NMR analysis.

Plates containing 10 ml of yeast agar (Sigma, 01497) were prepared before the addition of the TLC plate, which was faced upwards. Nine millilitres of Mueller-Hinton agar (see section 2.1.1) and 1 ml of 1.5 x 10^8 CFU suspension of sensitive strains of either *E. coli* or *S. aureus* (see section 2.1.3) in Ringers solution, were mixed and poured over the TLC plate. The plates were left in an incubator set at 37°C for 12 hours then coated in a 2.5 mg/ml solution of MTT (methyl thiazolyl tetrazolium) and allowed to incubate over 2 hours (Kagan *et al.,* 2014).

Results

The following 3 chapters discuss the results of both the APO, and the potentially pathogenic organisms isolated, from the 3 differing locations selected. This includes the New Forest, selected due to its classification as an ancient forest (section 3.0), as well Friston Forest (section 4.0), which is classified as a modern forest. Chapter 3, (section 5.0), discusses the results obtained from organisms isolated at Dawes Farm, providing a wider perspective on the effect a higher interaction rate of animals and humans have on the soil microbiome, and the hence resulting inhibitory activity seen.

3.0 Investigation of soil samples from the New Forest

3.0 Investigation of soil samples from New Forest

Sampling of ancient woodland took place at New Forest, Hampshire, for the cultivation of both potentially pathogenic bacteria, as well as APO. The New Forest is well known for the vast amounts of differing wildlife, including free-roaming deer, ponies and birds, as well as being a recognisable tourist attraction, all capable of affecting the soil microbiome (Forestry Commission, 2004). This consequently may affect the presence of organisms, including potential APO, within the soil, allowing the effects of an increased amount of human and animal interaction, and the forest's known great history, to be compared to the other sample locations. The New Forest is classed as being an 'ancient' forest, due to it originating before 1600 A.D, with it also mentioned in the Domesday book, completed in 1086, also confirming the forest was not established due to tree planting (National Archives, 2017; Spake *et al*., 2016).

Several different samples of the 219 square mile area of the New Forest, were taken and studied, in collaboration with the Forestry Commission, to increase the chances of isolating APO, as well as pathogenic bacteria, and to allow comparison between the samples collected at the New Forest, due to differing location characteristics.

3.1 Methods

3.1.1 Isolation and characterisation of microorganisms within the soil

Soil samples were collected at several varying areas of the New Forest using methods stated in section 2.2.1, with all locations and their characteristics noted (see Appendix Table A1). Isolation of pathogenic organisms from the non-dried (wet) soil samples, was carried out according to section 2.2.2.2, and characterised using the several biochemical tests as stated in section 2.2.3.2. Isolation of APO was carried out according to section 2.2.2.3, using potato starch agar only, and characterised as seen in section 2.2.3.3, which included DNA extraction for PCR based identification of *Streptomyces* spp., however PCR itself was not carried out due to time constraints, hence APO could only be presumed as belonging to this genus.

3.1.2 Further testing of the Antimicrobial Producing Organisms isolated

Perpendicular screening and the starvation method were carried out with sensitive strains of test bacteria, according to sections 2.2.4.2, and 2.2.4.5 respectively. *P. aeruginosa* and *P. mirabilis* were not further used in the starvation method, due to lack of inhibition towards these organisms in initial screening, and time constraints. The readdition of supernatants was carried out according to the methodology as written in 2.2.4.5, plates of sensitive strains of *E. coli*, *S.*

epidermidis and *S. aureus* were streaked and incubated 24 hours prior to the readdition of APO supernatants, stored from the previous round of the starvation method.

The several supernatants produced from isolate 26, over several of the differing days incubation in the starvation method (see above), were combined and further analysed due to the displayed antibacterial capabilities. Due to the inhibition of the same test organisms by isolate 26, the combined supernatants were assumed to contain the same compounds. Solvent extraction, TLC and NMR were carried out according to sections 2.2.5.1, 2.2.5.2. and 2.2.5.3. Note that NMR was run for both of the compounds separated from the acetonitrile fraction, with a Rf value of one, to check if they were the same compound.

The solvent systems were used as followed; chloroform: acetic acid (29:1) for the precipitate, chloroform: methanol (19:1) for both petroleum ether and dichloromethane fractions, and ethyl acetate: methanol: water (13:2:1) for the acetonitrile fraction.

3.2 Results

Forty-two soil samples were collected in November 2016 from differing areas of the New Forest, with Appendix Table A1 displaying the coordinates and sample area information.

3.2.1 Isolation of pathogenic microorganisms

Seventy-seven microorganisms were isolated and identified from the 42 non-dried soil samples, collected at the New Forest in November 2016 (Tables 3.1 and A1). One isolate (1.3%) was identified as belonging to *Salmonella* spp., three (3.9%) identified as belonging to the *Pseudomonas* spp. and two (2.6%) were identified as *S. epidermidis*, using the range of biochemical tests and API results. Cultivation of other isolates included *Streptomyces* spp. (11 isolates) (14.3%), *Acinetobacter* spp. (11 isolates) (14.3%), *Kurthia* spp. (10 isolates) (13.0%), and the *Bacillus* spp. (31 isolates) (40.3%), all shown to be prevalent at the New Forest (Table 3.1). *Kurthia* spp. was noted as above, due to its irregular shape of colony morphology resembling that of a ‘birds feather’.

Table 3.1: The Characteristics and presumptive identities of 77 isolates from the even numbered soil samples from the New Forest. (+VE) indicates a positive result, and (-VE) indicates a negative result to the individual biochemical tests used. A lack of growth seen by the individual organisms is also noted (No growth) Gram stain, oxidase, lactose fermentation, mannitol fermentation, catalase, citrate, indole, coagulase and urease results are detailed, as well as colony size and morphology for differing isolates. Colony morphology is noted as the organism is seen on nutrient agar. Coagulase tests were carried out on Gram-positive cocci only. Blank results in the biochemical test sections, indicate that the specific test was not carried out on the corresponding isolate. (API), indicates the presumptive spp. was determined using the API test as stated in section 2.1.2.

Isolate number	Colony size (mm)	Colony morphology	Gram Stain	Oxidase test	Lactose fermentation	Mannitol fermentation	Catalase	Simmon's Citrate test	Indole test	Coagulase test	Urease test	Presumptive spp.
2.1	3	Glossy, white, risen.	Long Gram-positive rods.	-VE			+VE	+VE	-VE			*Streptomyces* spp.
2.2	1	Cream, circular, matte.	Gram-negative rods.	+VE	+VE		+VE	+VE	-VE			*Pseudomonas* spp.
2.3	3	White, irregular shape, matte.	Gram-positive rods	-VE		-VE	+VE	+VE				*Bacillus* spp.
2.4	1	Cream, circular, matte.	Gram-negative rods.	-VE	-VE		+VE					*Acinetobacter* spp.
4.1	5	Cream, irregular shape, glossy.	Gram-negative rods.	-VE	+VE		+VE	-VE	-VE			*Cedecea* spp.
4.2	10	White, circular, matte.	Gram-positive rods.	-VE		No growth	+VE	+VE				*Bacillus* spp.
4.3	2	Yellow, circular, glossy.	Gram-negative rods.	-VE	-VE		+VE	-VE	-VE			*Acinetobacter* spp. (API)
4.4	<1	White, circular, glossy.	Gram-negative rods.	+VE	-VE		-VE	-VE	-VE			*Moraxella* spp.
6.1	<1	Pale white, circular, matte.	Gram-negative rods.	-VE	-VE		+VE	-VE			-VE	Shigella spp.
8.3	1	White, circular, matte.	Gram-positive rods	-VE			+VE	+VE	-VE			*Bacillus* spp.
8.4	<1	White, circular, matte.	Gram-negative rods.	+VE	+VE			+VE	-VE			*Acinetobacter* spp.
10.1	3	White, circular.	Gram-positive rods with spores.	+VE	-VE		+VE	+VE	-VE			*Bacillus* spp.
10.2	3	White, irregular shapes, matte.	Gram-positive rods with spores.	-VE			+VE	+VE				*Bacillus* spp.
10.3	3	White, irregular shape, matte.	Gram-positive rods with spores.	+VE			+VE	+VE				*Bacillus* spp.
10.4	1	White, circular, matte.	Gram-positive rods with spores.	+VE			+VE	+VE				*Bacillus* spp.

Isolate number	Colony size (mm)	Colony morphology	Gram Stain	Oxidase test	Lactose fermentation	Mannitol fermentation	Catalase	Simmon's Citrate test	Indole test	Coagulase test	Urease test	Presumptive spp.
12.1	4	White, glossy, circular-rough edges.	Long Gram-positive rods	+VE	-VE	-VE	+VE	+VE	-VE			*Streptomyces* spp.
12.2	2	Cream, circular, glossy.	Gram-positive rods.	+VE		+VE	+VE	+VE				*Bacillus* spp.
12.4	<1	White, glossy, circular.	Long Gram-positive rods.	+VE		-VE	+VE	+VE				*Streptomyces* spp.
12.5	7	White, circular, matte.	Gram-positive rods with spores.	-VE		+VE	+VE	+VE				*Bacillus* spp.
12.6	5	White, irregular shape with extensions, matte.	Gram-positive rods.	-VE		-VE	+VE	-VE				*Kurthia* spp.
12.7	2	Cream, circular, glossy.	Gram-negative rods.	+VE	+VE		+VE	+VE	-VE			*Pasteurella* spp. (API)
14.1	15	Cream, irregular shape with extensions, matte.	Gram-positive rods	-VE	-VE		+VE	-VE	-VE			*Kurthia* spp.
14.2	2	White, irregular shape, matte.	Long Gram-positive rods	+VE			+VE	+VE	-VE			*Streptomyces* spp.
14.3	4	White, irregular shape, matte.	Gram-negative rods.	+VE	-VE		+VE	-VE	-VE			*Moraxella* spp.
14.4.1	4	Large, glossy, circular.	Gram-negative rods.	-VE	-VE		+VE					*Acinetobacter* spp. (API)
14.4.2	4	White, circular, glossy.	Gram-negative rods.	-VE	-VE		+VE					*Acinetobacter* spp. (API)
14.6	1	White, circular, glossy.	Long Gram-positive rods	-VE	-VE		+VE	+VE	-VE			*Streptomyces* spp.
14.7	1	White, circular, glossy.	Long Gram-positive rods	-VE	-VE		+VE	+VE	-VE			*Streptomyces* spp.
16.1	15	White, irregular shape, matte.	Gram-positive rods.	-VE	-VE	-VE	+VE	-VE				*Kurthia* spp.
16.2	5	White, rough-edged circular, matte.	Gram-positive rods.	-VE	No growth	No growth	+VE	+VE				*Bacillus* spp.
16.3	2	Cream, circular, glossy.	Gram-negative rods.	-VE	-VE		+VE	+VE			-VE	*Salmonella* spp.
16.5	<1	White, circular, glossy.	Long Gram-positive rods	+VE	No growth				-VE			*Streptomyces* spp.
16.6	4	Cream, circular, glossy.	Gram-negative rods.	+VE	-VE		+VE	+VE	-VE			*Pseudomonas* spp.
18.1	30	Cream, irregular shape with extensions, matte.	Gram-positive rods.	-VE		-VE	+VE	-VE				*Kurthia* spp.
18.3	4	Cream, circular, glossy.	Gram-negative rods.	+VE	-VE			-VE	-VE			*Moraxella* spp.
18.4	2	Small, white colonies.	Gram-negative rod	+VE	+VE		+VE	+VE	-VE			*Pasteurella* Spp. (API)
18.5.1	3	Matte, rough circular.	Gram-positive rods.	-VE	+VE		+VE	+VE	-VE			*Bacillus* spp.
18.5.2	2	Glossy, circular.	Gram-positive rods.	+VE	+VE	-VE	+VE	+VE	-VE			*Bacillus* spp.

Isolate number	Colony size (mm)	Colony morphology	Gram Stain	Oxidase test	Lactose fermentation	Mannitol fermentation	Catalase	Simmon's Citrate test	Indole test	Coagulase test	Urease test	Presumptive spp.
18.5.3	3	White, circular, glossy.	Gram-negative rods.	-VE	-VE		+VE					*P. paucimobilis* (API)
18.6	<1	White, circular, glossy.	Gram-negative rods.	-VE	-VE		+VE					*Acinetobacter* spp. (API)
20.1	12	White, irregular shape, matte.	Gram-positive rods.	-VE		-VE	+VE	-VE				*Kurthia* spp.
20.2	8	White, circular, matte.	Gram-positive rods.	-VE		-VE	+VE	+VE				*Bacillus* spp.
20.4	2	Cream, circular, glossy.	Long Gram-positive rods.	+VE			+VE	+VE	-VE			*Streptomyces* spp.
20.5	4	Cream, irregular shape, glossy.	long Gram-positive rods.	+VE		-VE	+VE	+VE				*Streptomyces* spp.
20.6	2	White, irregular shape, matte.	Gram-positive rods.	-VE	-VE	-VE	+VE	-VE	-VE			*Kurthia* spp.
20.7	1	Yellow, circular, glossy.	Gram-negative rods.	-VE	+VE	No growth	+VE	-VE	-VE			*Acinetobacter* spp. (API)
20.9	<1	White, circular, matte.	Gram-positive rods.	-VE			+VE	+VE				*Bacillus* spp.
22.1	1	White, matte, irregular shape.	Gram-positive rods with spores.	-VE		+VE	+VE	+VE				*Bacillus* spp.
22.2	2	White, circular, matte.	Gram-positive rods with spores.	-VE	+VE	+VE	+VE	+VE				*Bacillus* spp.
22.3.1	2	Cream, irregular shape, matte.	Gram-positive rods with spores.	-VE		+VE	+VE	+VE				*Bacillus* spp.
22.3.2	1	White, little, circular.	Gram-positive rods with spores.	-VE		+VE	+VE	+VE				*Bacillus* spp.
24.1	12	White, irregular shape with extensions, matte.	Gram-positive rods.	-VE		-VE	+VE	-VE				*Kurthia* spp.
24.3	3	White, circular, matte.	Gram-negative rods.	-VE	-VE		+VE	-VE	-VE			*Acinetobacter* spp. (API)
24.4	<1	White, circular, glossy	Gram-negative rods.	-VE	-VE			-VE	-VE			*Acinetobacter* spp.
26.1	1	Grey, glossy.	Gram-positive rods with spores.	+VE		+VE	+VE	+VE				*Bacillus* spp.
26.2	1	Cream, glossy.	Gram-positive rods with spores.	+VE		+VE	+VE	+VE				*Bacillus* spp.
26.3.1	1	White, circular, matte.	Gram-positive rods with spores.	+VE		+VE	+VE	+VE				*Bacillus* spp.
26.3.2	1	Grey, glossy.	Gram-positive rods.	+VE		+VE	+VE	+VE				*Bacillus* spp.
26.3.3	1	Small, white, glossy.	Gram-positive cocci.	-VE		-VE	+VE	+VE		-VE		*Staphylococcus epidermidis*
26.4	2	Grey, small, glossy.	Gram-negative rods.	-VE	+VE		+VE	+VE	-VE			*Acinetobacter* spp. (API)
28.1	10	White, circular with rough edges, matte.	Gram-positive rods.	+VE		-VE	+VE	+VE				*Bacillus* spp.

Isolate number	Colony size (mm)	Colony morphology	Gram Stain	Oxidase test	Lactose fermentation	Mannitol fermentation	Catalase	Simmon's Citrate test	Indole test	Coagulase test	Urease test	Presumptive spp.
28.2	4	White, irregular shape, matte.	Long Gram-positive rods	+VE		-VE	-VE	+VE				*Streptomyces* spp.
28.3	1	Cream, circular, glossy.	Long Gram-positive long rods	+VE	+VE			+VE	-VE			*Streptomyces* spp.
28.4	1	White, circular, glossy	Gram-negative rods.	-VE	-VE		+VE					*Pasteurella* spp. (API)
30.1	2	White, circular, matte.	Gram-positive rods with spores.	+VE		+VE	+VE	+VE				*Bacillus* spp.
30.2.1	1	White, circular, glossy.	Gram-positive cocci.	-VE		-VE	+VE	+VE		-VE		*Staphylococcus epidermidis*
30.2.2	1	Pale white, small, circular.	Gram-positive rods with spores.	+VE		-VE	+VE	+VE				*Bacillus* spp.
32.1	3	White, circular with rough edges, matte.	Gram-positive rods with spores.	+VE	-VE		+VE	+VE				*Bacillus* spp.
34.3	4	White, circular with extensions, matte.	Gram-positive rods in chains.	-VE		No growth	+VE	+VE				*Kurthia* spp.
36.1	25	Cream, irregular shape with extensions, matte.	Gram-positive rods in chains.	-VE		No growth	+VE	+VE				*Kurthia* spp.
36.2	5	Cream, circular, glossy.	Gram-negative rods.	+VE	-VE			+VE	-VE			*Acinetobacter* spp. (API)
36.3	6	White, circular with extensions, matte.	Gram-positive rods in chains.	-VE		No growth	+VE	+VE				*Kurthia* spp.
36.4	<1	White, circular, matte.	Gram-positive rods.	+VE	+VE			+VE	-VE			*Bacillus* spp.
38.1	1	Cream, circular, matte.	Gram-positive rods with spores.	+VE		+VE	+VE	+VE				*Bacillus* spp.
38.2	1	Grey, circular, matte.	Gram-positive rods with spores.	+VE	No growth							*Bacillus* spp.
38.4	1	White, circular, matte.	Gram-positive rods with spores.	+VE			+VE	+VE				*Bacillus* spp.
42.1	<1	White, circular, matte.	Gram-positive rods with spores.	+VE		+VE	+VE	+VE				*Bacillus* spp.

3.2.2 Identification of *Streptomyces* spp. from APO isolated

One out of the 34 (2.9%) APO isolated using dried soil samples, isolate 20, was identified as potentially belonging to the *Streptomyces* spp. due to Gram stain (see appendix Table A2), and colony morphology representative of the genus. DNA extraction however resulted in too low a value for DNA concentration, and hence due to time constraints, the DNA was not tested using determinative PCR, meaning the genus of isolate 20 could not be confirmed. This was also the case for the 11 *Streptomyces* isolates identified from the wet soil samples (Table 3.1).

3.2.3 Testing of Antimicrobial Producing Organisms

3.2.3.1 Novel antimicrobial screening against sensitive organisms

Thirty-four organisms with the ability to produce inhibitory compounds were isolated using potato starch agar, from the initial collection of samples from New Forest (See appendix table A2). All 34 isolates, including the potential *Streptomyces* spp., were then subjected to further testing to determine the range of inhibitory activity using several test organisms; sensitive strains of *E. coli*, *S. epidermidis*, *S. aureus*, *P. mirabilis* and *P. aeruginosa* were used for initial screening of the APO. Different isolates showed a range of different activity against both Gram-positive and Gram-negative bacteria, however no inhibition against *P. aeruginosa* or *P. mirabilis* was seen. Five isolates displayed inhibitory ability from initial perpendicular screening (14.7%); isolates 9, 10, 11, 16 and 26 showed inhibition at varying temperatures (Table 3.2). Isolates 9 and 10 displayed the largest range of activity, with inhibition seen against *S. epidermidis*, *S. aureus*, and *E. coli* after being incubated at 25°C for 2 days (Figures 3.1 and 3.2). Isolate 11, also isolated at location 16, showed inhibition only against *E. coli* after both 0 and 2-day incubation at 25°C. Isolate 26 showed ability to produce inhibition under a range of conditions against both *S. epidermidis* and *S. aureus* due to repeated inhibition at both 25°C and 37°C after both 0 and 2-day incubation (Figure 3.3). Isolate 16 also showed inhibition against *S. epidermidis* and *S. aureus*, with 2-day incubation at both 25°C and 37°C (Figure 3.4). Zero-day incubation of isolate 16 at 37°C showed inhibition against *S. epidermidis* and *S. aureus* (Figure 3.4), with 0-day incubation at 25°C only showing inhibition towards *S. aureus*.

Table 3.2: The inhibitory activity shown by five of the 34 isolates, collected from the New Forest in November 2016, using the perpendicular screening method. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
9	-	*S. epidermidis*, *S. aureus*, and *E. coli*	-	-
10	-	*S. epidermidis*, *S. aureus*, and *E. coli*	-	-
11	*E. coli*	*E. coli*	-	-
16	*S. aureus*	*S. epidermidis* and *S. aureus*	*S. epidermidis* and *S. aureus*	*S. epidermidis* and *S. aureus*
26	*S. epidermidis* and *S. aureus*.	*S. epidermidis* and *S. aureus*.	*S. epidermidis* and *S. aureus*.	*S. epidermidis* and *S. aureus*.

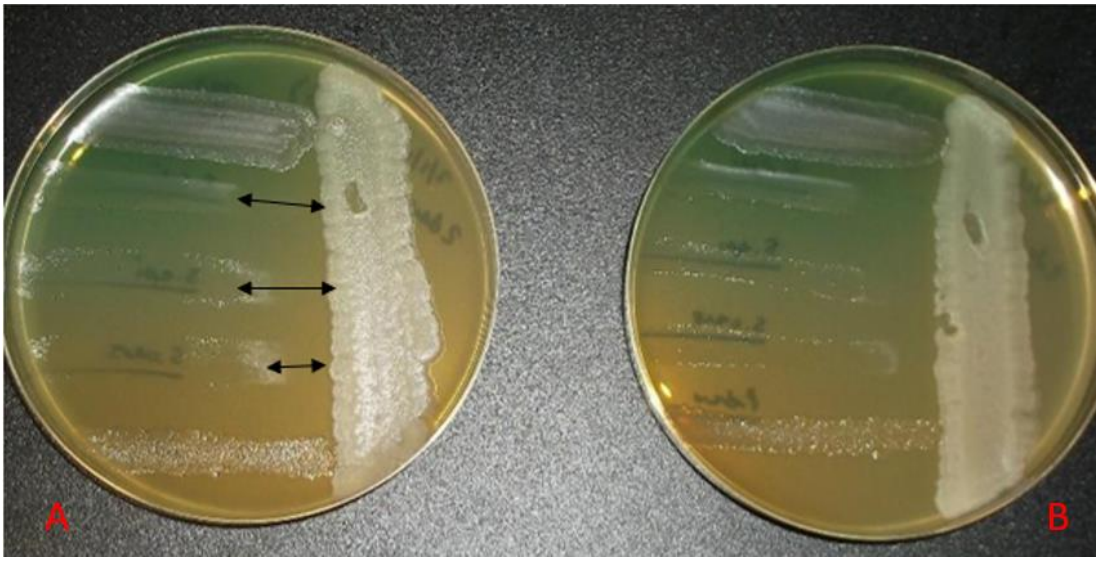

Figure 3.1: Isolate 9 grown for 2 days at 25°C, using the perpendicular screening method, displaying inhibition towards *E. coli*, *S. epidermidis*, and *S. aureus,* in duplicate (A and B). Test bacteria from top to bottom, *P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus* and *P. mirabilis*. The arrows displayed on duplicate A indicate the inhibition of test bacterial growth away from the APO being tested (9).

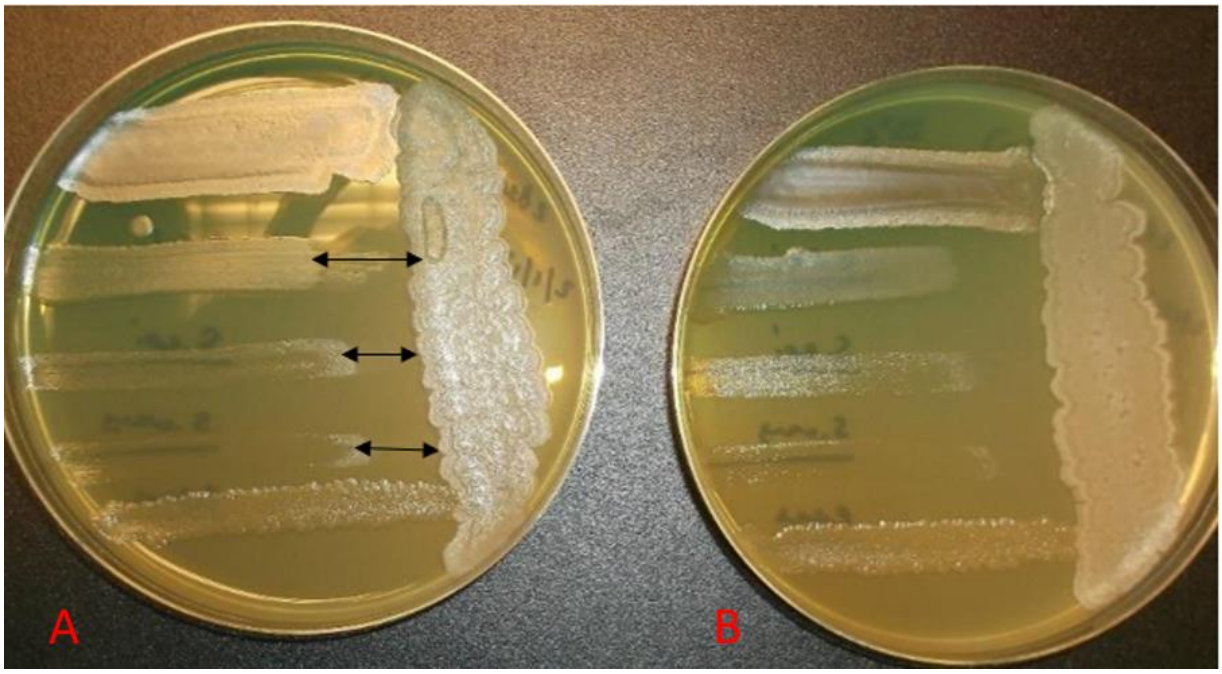

Figure 3.2: Isolate 10 grown for 2 days at 25°C, displaying inhibition in the perpendicular screening method towards *E. coli*, *S. epidermidis*, and *S. aureus,* in duplicate. Test bacteria from top to bottom, *P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus* and *P. mirabilis*. The arrows on duplicate A indicate the extent of inhibition caused by APO 10.

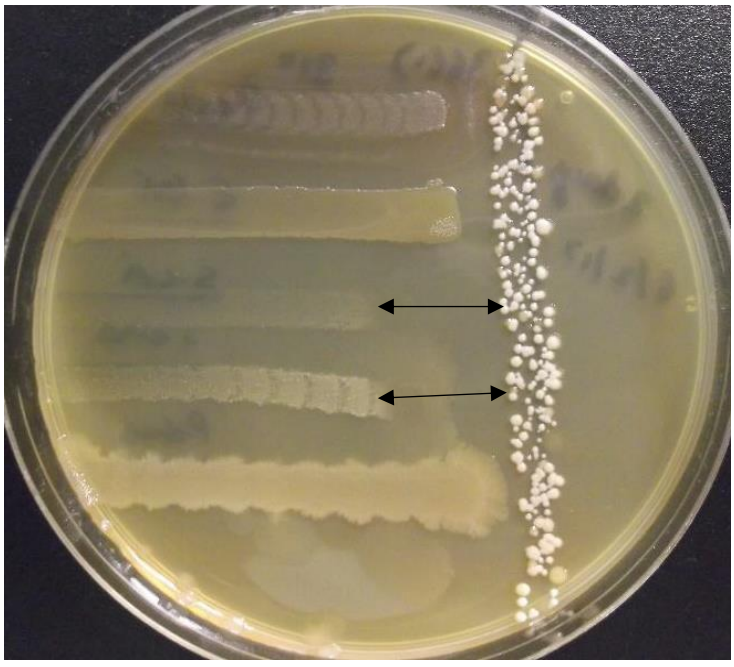

Figure 3.3: Isolate 26 incubated for 2 days at 37°C, displaying inhibition against *S. epidermidis* and *S. aureus* in the perpendicular screening method, indicated by the black arrows. Test bacteria from top to bottom, *P.aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus* and *P. mirabilis*.

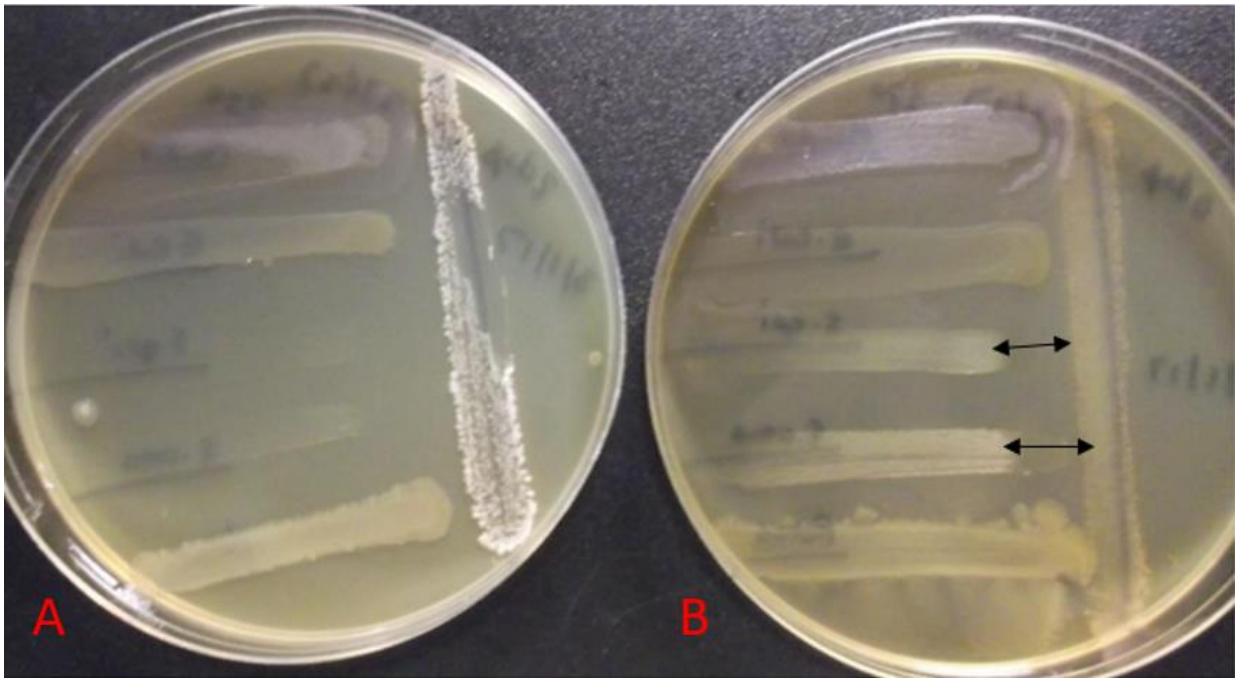

Figure 3.4: Isolate 16 incubated for 2 days at 25°C (plate A) and incubated for 0 days at 37°C (plate B), using the perpendicular screening method, with both displaying inhibition towards *S. epidermidis* and *S. aureus* indicated by the black arrows on plate B. No inhibition seen to *P. aeruginosa, E. coli* and *P. mirabilis*. Test bacteria from top to bottom, *P. aeruginosa*, *E. coli*, *S. epidermidis*, *S. aureus* and *P. mirabilis*.

3.2.3.2 Starvation method

The starvation method was used for further screening of the inhibitory activity of the APO. Twelve of the 34 (35.3%) isolates showed inhibition against one or more of the test bacteria used (Table 3.3); 5, 6, 13, 18, 27, 28, 29, 30, 31 and 34, as well as 16 and 26 which previously displayed inhibition in initial perpendicular screening, showed inhibition against at least one of the test bacteria used (*E. coli*, *S. aureus* or *S. epidermidis*) in the starvation method (Tables 3.2 and 3.3). Figures 3.5 and 3.6 display the inhibition against *E. coli* on plate count agar, by isolates 6 and 13 respectively, after both were incubated for 10 days at 25°C. Figure 3.7 displays the inhibition seen towards both *S. epidermidis* and *S. aureus* on plate count agar, by isolate 26 after incubation for 7 days at 37°C.

Table 3.3: Results from the first run of the starvation method using New Forest isolates, displaying APO with the ability to produce inhibition against the test bacteria at different incubation times (7, 10 and 14 days) and temperatures. The agar result in which inhibition was displayed on, is recorded in brackets; PC – plate count agar, MH – Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Days of incubation of isolate at 25°C- Inhibition seen - (agar result in bracket)**			**Days of incubation of isolate at 37°C- Inhibition seen - (agar result in bracket)**		
	7 Days	10 Days	14 Days	7 Days	10 Days	14 Days.
5	-	-	*E. coli*, *S. epidermidis* and *S. aureus*. (PC).	-	-	-
6	*S. epidermidis* and *S. aureus* (MH) *E. coli* (PC)	*E. coli* (both)	*S. epidermidis* and *S. aureus*, (PC).	-	-	-
13	*E. coli, S. epidermidis* and *S. aureus* (PC)	*E. coli* (MH and PC)	-	-	-	-
16	-	-	*S. aureus* and *S. epidermidis* (PC)	-	-	-
18	-	-	-	-	-	*S. epidermidis* and *S. aureus* (PC)
26	*S. epidermidis* and *S. aureus* (PC)	-	*S. aureus* (PC)	*S. epidermidis* and *S. aureus* (PC)	*S. aureus* (PC)	*S. epidermidis* and *S. aureus* (PC)
27	-	*S. epidermidis* and *S. aureus* (MH)	-	-	-	-
28	-	-	-	-	*S. aureus* (MH and PC)	-
29	-	-	-	-	*S. aureus* (PC)	-
30	-	*S. epidermidis* and *S. aureus* (MH)	-	-	-	-
31	-	*E. coli* (PC)	-	-	-	-
34	-	*E. coli* and *S. epidermidis* (PC).	-	-	-	-

Figure 3.5: Isolate 6 that was isolated from New Forest, and incubated for 10 days at 25°C, before displaying inhibition against *E. coli* streaked onto plate count agar, from the initial starvation method.

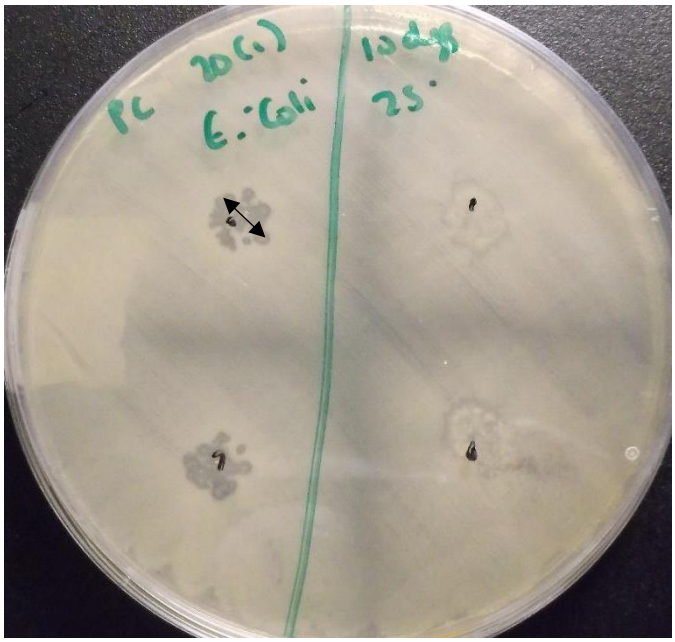

Figure 3.6: Isolate 13 initially isolated from New Forest, displaying inhibition against *E. coli* on plate count agar, after incubation for 10 days at 25°C, using the starvation method.

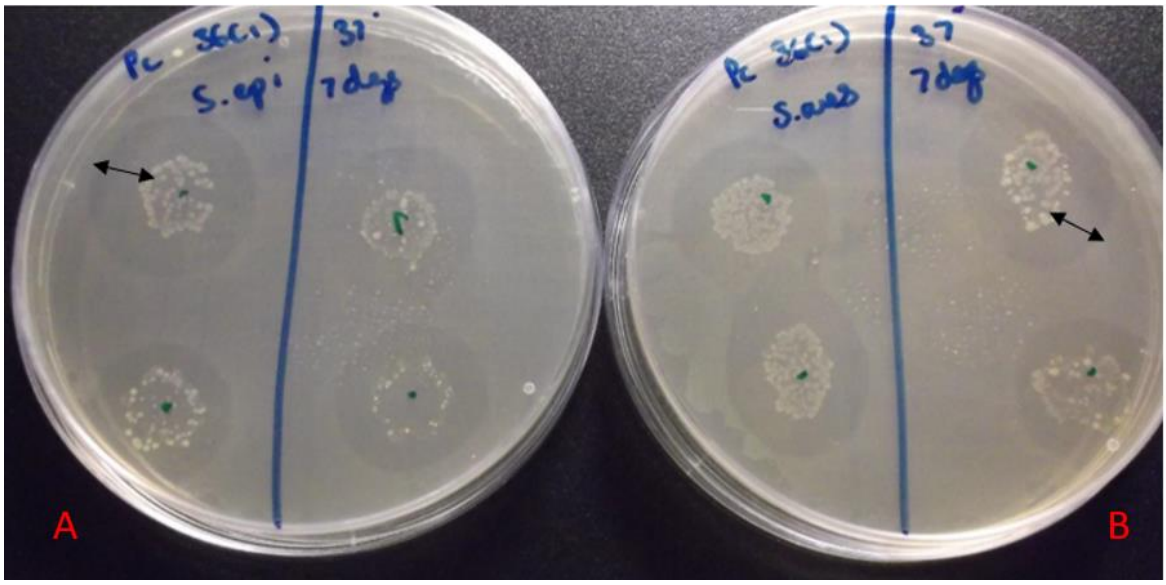

Figure 3.7: Displays the inhibition seen towards test bacteria *S. epidermidis* and *S. aureus* on plate count agar, after New Forest isolate 26 was incubated for 7 days at 37°C in the starvation method. The two arrows detail the diameter of the inhibition presented by isolate 26.

The starvation method was repeated using the supernatant of isolates that produced inhibition prior, which was stored at 4°C and collected from the first round of testing. Results were seen to differ from that of the original starvation method data, when the supernatant was added directly to the test bacteria, after the centrifugation of incubated samples (Table 3.4). Isolates 5, 6, 13, 27, 28, 29, 30, 31 and 34 displayed less/no inhibition than seen prior. Isolate 26 was shown to display the same level of inhibitory activity and isolates 16 and 18 displayed higher amounts of inhibitory action than seen previously towards the *Staphylococcus* spp. and *E. coli* respectively.

The reincubation of the New Forest APO that had previously shown inhibition in the starvation method, detailed differing results (Table 3.5). Isolates 5, 6, 13, 16, 18, 26, 28, 29, 30, 31 and 34 all showed less range of inhibitory activity and/or less inhibition towards test bacteria than previously (Table 3.3). Near identical inhibition was seen by isolate 26 with inhibitory activity displayed at similar incubation times and temperatures. Isolates 27, 28, 29, 30, 31 and 34 displayed no evidence of inhibition in either duplicates of the starvation method (Tables 3.4 and 3.5).

Table 3.4: Inhibition seen from the reuse of the supernatants obtained from the first round of the starvation method using prolonged incubation of the New Forest APO. Note that N/A indicates that a supernatant was not available for repeat testing. The agar in which inhibition was seen, is placed in brackets; PC – plate count agar, MH – Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Days of incubation of isolate at 25°C - Inhibition seen (agar result in bracket)**			**Days of incubation of isolate at 37°C – inhibition seen (agar result in bracket)**		
	7 days	**10 days**	**14 days**	**7 days**	**10 days**	**14 days**
5	-	-	*E. coli* (PC)	-	-	-
6	-	*S. epidermidis* (PC)	-	-	-	-
13	N/A	*E. coli, S. epidermidis* and *S. aureus* (PC)	N/A	N/A	-	N/A
16	*S. epidermidis* and *S. aureus* (PC)	N/A	N/A	*S. epidermidis* and *S. aureus* (PC).	N/A	*S. epidermidis* and *S. aureus* (PC).
18	*E. coli* (PC)	N/A	*S. epidermidis* an *E. coli* (PC).	N/A	N/A	-
26	*S. epidermidis* and *S. aureus* (PC).	N/A	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and S. aureus (PC).	N/A	*S. epidermidis* and *S. aureus* (PC).
27	-	N/A	-	-	N/A	-
28	N/A	-	-	N/A	-	-
29	N/A	-	-	-	N/A	-
30	-	N/A	-	-	N/A	-
31	-	-	-	-	-	-
34	-	-	-	-	-	-

Table 3.5: Inhibition displayed in the repeat of the whole starvation method, using prolonged incubation at varying days (7, 10 and 14 days), of New Forest APO that had shown inhibition previously. The agar in which inhibition was seen is presented in brackets; PC – plate count agar, MH – Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Days of incubation of isolate at 25°C- Inhibition seen (agar result in bracket)**			**Days of incubation of isolate at 37°C – Inhibition seen (Agar result in bracket)**		
	7 days	**10 days**	**14 days**	**7 days**	**10 days**	**14 days**
5	-	-	-	-	*S. epidermidis* (MH)	-
6	-	-	-	-	-	-
13	-	-	-	-	-	-
16	-	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and *S. aureus* (PC).	-	*S. epidermidis* and *S. aureus* (PC).	*S. aureus* (PC).
18	-	-	-	-	-	-
26	*S. aureus* and *S. epidermidis* (MH) and *S. aureus* (PC).	-	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* (PC).	*S. epidermidis* (PC).
27	-	-	-	-	-	-
28	-	-	-	-	-	-
29	-	-	-	-	-	-
30	-	-	-	-	-	-
31	-	-	-	-	-	-
34	-	-	-	-	-	-

3.2.3.3 Chemical analysis of potential antimicrobial compounds- pilot study

Solvent extraction and TLC, for the elucidation of separate compounds was carried out using supernatant collected from isolate 26, due to the broad conditions in which inhibitory activity was produced by this APO, towards the test organisms used. Several of the supernatants produced over the differing days incubation in the starvation method, were combined for chemical analysis (see section 3.2.3.2). The compounds and their rf values resulting from TLC, using the precipitate as well as solvents petroleum ether, dichloromethane and acetonitrile, that were used for solvent extraction, can be seen in Tables 3.6, 3.7, 3.8 and 3.9 respectively. These tables also display compounds with seen antibacterial activity against the test bacteria used (*E. coli* and/or *S. aureus)*; only three compounds were seen to present inhibitory activity against *S. aureus* only, with two extracted using dichloromethane, and one of the compounds extracted using acetonitrile (Tables 3.8 and 3.9). These compounds were then placed through NMR, however the

structures of the inhibitory compounds were not determined due to time constraints (see Appendix figures A1, A2, A3, A4, A5, A6, A7 and A8).

Table 3.6: The Rf values of the three compounds separated using TLC from the precipitate of the supernatant of isolate 26. The solvent system used in TLC consisted of a 29:1 ratio of chloroform: acetic acid. +VE = compound displayed antimicrobial capability, -VE = no antimicrobial capability of the compound was seen. Methods and data was collected from the work carried out by Mr Rob Warren, and Dr Adam LeGresley.

Precipitate	**Rf**	**Antimicrobial activity**
Compound 1	1	-VE
Compound 2	0.71	-VE
Compound 3	0.04	-VE

Table 3.7: The compounds and their subsequent Rf values and the antimicrobial capabilities of isolate 26's supernatant extracted using petroleum ether. The solvent system used consisted of a 19:1 ratio of chloroform: methanol. +VE = compound displayed antimicrobial capability, -VE = no antimicrobial capability of the compound was seen. Methods and data was collected from the work carried out by Mr Rob Warren, and Dr Adam LeGresley.

Petroleum ether	**Rf**	**Antimicrobial activity**
Compound 1	1	-VE

Table 3.8: The Rf values and antimicrobial capabilities of the three compounds extracted from the supernatant of isolate 26 using dichloromethane. The solvent system used consisted of a 19:1 ratio of chloroform: methanol. +VE = compound displayed antimicrobial capability, -VE = no antimicrobial capability of the compound was seen. Methods and data was collected from the work carried out by Mr Rob Warren, and Dr Adam LeGresley.

Dichloromethane	**Rf**	**Antimicrobial activity**
Compound 1	1	+VE (*S. aureus*)
Compound 2	0.68	+VE (*S. aureus*)
Compound 3	0.58	-VE

Table 3.9: The Rf values of the compounds that were extracted using two TLC plates, for the separation of the acetonitrile fractions of the supernatant originating from isolate 26. The solvent system consisted of a ratio of 13:2:1 of ethyl acetate: methanol: water. +VE = compound displayed antimicrobial capability, -VE = no antimicrobial capability of the compound was seen. Methods and data was collected from the work carried out by Mr Rob Warren, and Dr Adam LeGresley.

Acetonitrile	**Rf**	**Rf**	**Antimicrobial activity**
Compound 1	1	1	+VE (*S.aureus*)
Compound 2	0.74	(missing)	-VE
Compound 3	0.68	0.63	-VE
Compound 4	0.58	0.55	-VE
Compound 5	0.25	0.34	-VE
Compound 6	0.18	0.12	-VE
Compound 7	0.05	0.03	-VE

3.3 Discussion

The New Forest selected due to its classification as an ancient forest, notably different from the other two sample locations, proved to be successful in the isolation of APO. Thirty-four APO were isolated and further tested, with five (14.7%) of these displaying some form of inhibitory activity towards either *E. coli*, *S. epidermidis*, and/or *S. aureus* in initial perpendicular screening (Table 3.2). Isolates 16 and 26 showed great activity at a range of temperatures (25°C and 37°C) after both 0 and 2-day incubation using this perpendicular screening method, with both APO continuing to produce this inhibition under the range of conditions used in the differing repeats of the starvation method (Tables 3.3, 3.4 and 3.5). Despite the work to lower the amounts of Gram-negative organisms in the soil samples by drying, both these organisms were seen to be Gram-negative rods, which upon further investigation as future work, may explain their inhibitory activity towards the Gram-positive organisms only, and their ability to produce inhibitory compounds under a range of conditions, compared to the other APO with inhibitory ability (Table A2).

The starvation method, used to induce inhibitory activity by the APO using the creation of stressful environments, displayed higher amounts of inhibitory activity compared to perpendicular screening, with 12 (35.3%) organisms displaying some form of inhibitory action to the test bacteria used, over double the amount seen in initial screening (14.7%) (Tables 3.2 and 3.3). However, the repeat of the whole method using the APO that previously showed inhibition, only detailed three (8.8%) of these isolates with repeated inhibitory activity, showing a great reduction between the repeats of this method (Tables 3.3 and 3.5); isolates 6, 13, 18, 27, 28, 29, 30, 31, and 34 showed no inhibitory activity in the repeat of the whole method (Table 3.5). As well as this, isolate 5 displayed less inhibition towards test bacteria, also presenting this inhibition on differing agars and at different temperatures; in the first run of the starvation method, isolate 5 displayed inhibition to both the *Staphylococcus* spp. and *E. coli* on plate count agar at 25°C, however upon the repeat, inhibition was seen against *S. epidermidis* only, on Mueller-Hinton agar, after incubation at 37°C (Tables 3.3 and 3.5). This large decrease of activity, by APO previously shown with the ability to produce inhibitory compounds, may provide evidence that specific environmental/stress conditions are required to be met, before the activation of a series of complex pathways, that leads to the production of the antimicrobial compounds of interest (Figure 3.8) (Rokem *et al*., 2007). The conditions, however, were kept the same for both the repeats of the experiment, and hence this may further show the sensitivity for the activation of the genes involved in the production of antibiotics (Liu *et al*., 2013).

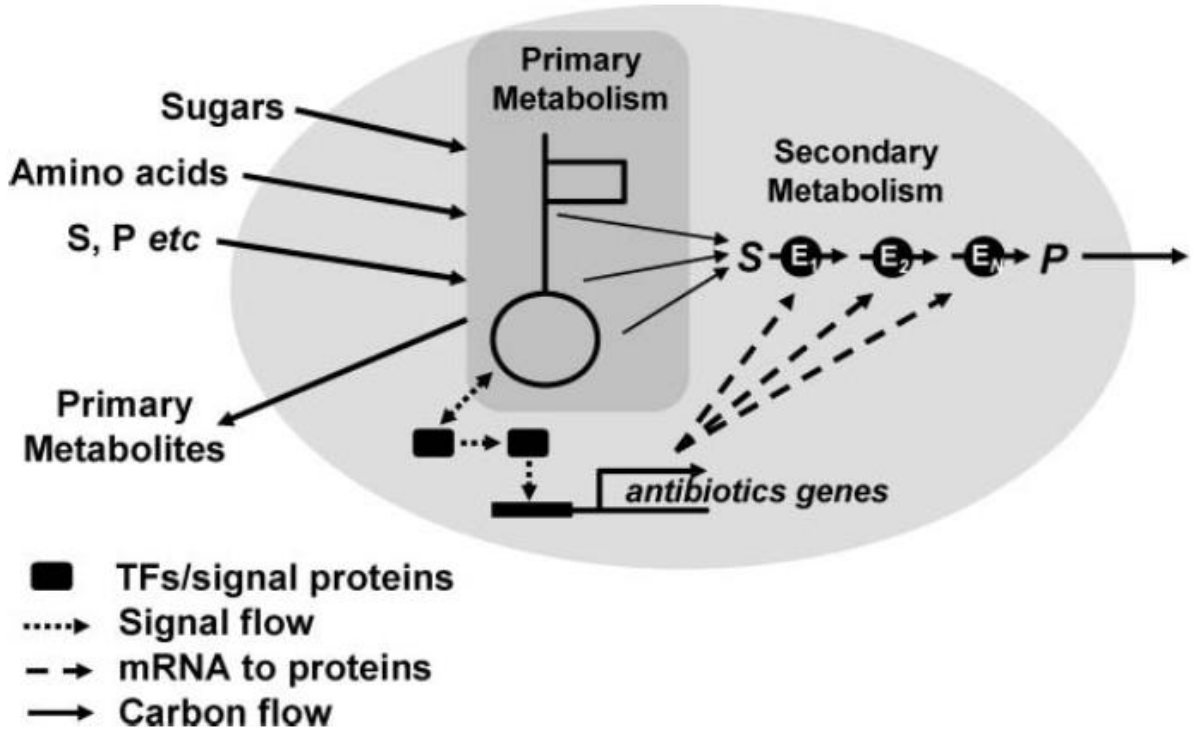

Figure 3.8: The several steps, and proteins required for the production of antibiotics from the secondary metabolism in microorganisms. Several different regulatory structures are detailed from primary to secondary metabolism, that control potential antibiotic production (Taken from Rokem *et al.,* 2007).

Supernatants collected and stored at 4°C from the first round of testing using the starvation method, were reapplied to plates of test bacteria according to the method used previously (detailed in section 2.2.4.5). Results from the readdition of the several supernatants displayed six of the 12 APO to have continued inhibitory activity with the use of at least one of the supernatants collected and retested. Isolates 16 and 18 were shown to display more inhibitory activity than seen previously, with isolate 16 seen to inhibit the same test bacteria as seen in the initial starvation method, however inhibition was further seen at both 25°C and 37°C upon the reuse of supernatant. The supernatant collected from isolate 18, displayed inhibition at 25°C, as well as showing additional inhibition to *E. coli*, compared to the inhibitory effects seen at 37°C only, in the initial starvation method (Tables 3.3 and 3.4). However, these results again detail a decrease in the amount of inhibitory activity seen compared to the initial method, with less/no inhibitory activity of supernatants produced by those that had formerly shown inhibition; isolates 6, 13, 27, 28, 29, 30, 31 and 34 all showed less inhibition compared to the initial addition of the supernatant (Tables 3.3 and 3.4). This

decrease in inhibitory activity may be due to the degradation of the antimicrobial compounds, which may be resultant of the time, and conditions, at which the collected supernatants were stored. Further work may include increasing the volume of the supernatant stored, as well as storage at differing conditions, to ensure the compounds remain viable. However, it was noted that some of the supernatants were unavailable for retesting, and hence this has a profound effect on the results (Table 3.4). If this method was also to be improved, direct analysis of the supernatant using DOSY-NMR, before it is placed onto test bacteria, would be used to allow determination of any compounds present, preventing the loss of any that maybe potentially useful.

The pilot study used in the present study is an example of this analysis. The supernatant of isolate 26, collected from the starvation method was further analysed due to its seen inhibition in this method, as well as initial perpendicular screening (Tables 3.2 and 3.3). The TLC bioassay detailed three of the separated compounds to have activity against *S. aureus* with two extracted using the solvent dichloromethane, and one extracted using acetonitrile (Tables 3.8 and 3.9). However, no inhibition was seen by the separated compounds towards *E. coli*, which further shows the lack of activity by isolate 26 towards this organism, previously seen in both the perpendicular screening and starvation method (Tables 3.2 and 3.3). Nevertheless, the three compounds were further analysed using NMR, however due to time constraints, confirmation of the compound structures could not be drawn (see Appendix figures A1, A2, A3, A4, A5, A6, A7 and A8). This hence highlights further work, which may include methods to aid in the identification of compounds such as mass spectrometry, to identify the molecular weight. As well as this, the continuation of this pilot study using other supernatants, collected from New Forest APO with displayed inhibitory ability from the starvation method, would increase the likelihood for the identification of novel antimicrobial compounds.

As well as working to isolate potential APO, this study also worked to cultivate organisms belonging to the *Streptomyces* spp., due to their known ability to produce a vast number of secondary metabolites, including antibiotics (Selvameenal *et al*., 2009; Miao and Davies, 2010). However, only one organism from the APO isolated from New Forest, was identified as potentially belonging to the *Streptomyces* spp., which was not confirmed due to time constraints preventing the PCR procedure for the identification of this genus, being carried out. This may highlight the New Forest as a location not favoured by the *Streptomyces* spp., which may be due to the soil conditions and competition of other organisms within the same soil microbiome, however samples areas were sparsely taken, and the lack of

Streptomyces in the soil samples collected, may not be representative of the *Streptomyces* population of the New Forest area as a whole. However, this is thought to be the first study aiming to isolate *Streptomyces* spp. and APO from the New Forest area, and hence further work focussing on the isolation of this genus would be preferable. The lack of *Streptomyces* spp. may also be due to the culture methods; potato starch agar only, was used for the cultivation of APO/ *Streptomyces* spp. compared to both the Friston Forest and Dawes Farm methods, for which both potato starch and oat agar were used to isolate APO.

However, from the wet soil isolates, 11 of the total 77 organisms isolated were presumptive *Streptomyces* spp. due to Gram stain and morphology (Table 3.1). Of the previously selected bacteria, only one organism (1.3%), was identified as belonging to the *Salmonella* spp., with no further organisms cultivated from the New Forest, identified as belonging to one of the genus' previously selected as of importance. Three (3.9%) and two (2.6%) isolates however were identified as belonging to the *Pseudomonas* spp. and as *S. epidermidis* respectively, with the remaining majority of isolates belonging to more known soil bacteria including 11 (14.3%) and 10 (13.0%) belonging to the *Acinetobacter* and *Bacillus* genus' respectively, as well as a large amount characterised as belonging to the *Kurthia* spp. (40.3%) (Table 3.1). The cultivation of these supposedly pathogenic bacteria was carried out to characterise the sample area, as well as to compare to the presence, or lack of APO from the same location, however, due to lack of isolation of the previously selected bacteria, extensive comparison cannot be conducted. However, the *Salmonella* spp. was isolated from sample site 16, as were the APO 9, 10 and 11, that showed inhibitory activity in initial perpendicular screening (Table 3.2), which may indicate a correlation between the presence of APO and pathogenic organisms, nevertheless further work sampling the same and differing areas of the New Forest would be required to further prove this hypothesis. This lack of pathogenic organisms may show their lack of presence in the soil, or the organisms themselves maybe hard to cultivate, due to the conditions, and/or the competition between the other bacteria in the same sample.

The New Forest has proven to be successful in the isolation of APO from the sites sampled, due to the inhibition seen in both perpendicular screening and the starvation method. However further work/sampling is necessary for the isolation of higher amounts of APO, and organisms belonging to the *Streptomyces* spp. Future work could also include the confirmation of isolates belonging to the *Streptomyces* spp., using PCR, of both the organisms isolated in the present study, and those isolated from any further sampling.

4.0 Investigation of soil samples from Friston Forest

4.0 Investigation of soil samples from Friston Forest

Friston Forest, another known national forest, is located in East Sussex, and differs from the ancient New Forest, due to the more recent planting of trees in the 1950s, classifying the forest as modern (Sussex Wildlife Trust, 2017). As a result, Friston Forest is less diverse in regard to tree type, with this forest largely made up of pine trees, which hence may affect the ecosystem that flourishes there, compared to that of the New Forest, allowing the potential for differing organisms, including both pathogenic and antimicrobial producing bacteria, to be isolated. As with the New Forest, the Forestry Commission are in charge of managing the 688 acres of Friston Forest, which it is also known to be a hotspot for tourists and mountain biking (Woodland Trust, 2017; Forestry Commission, 2017).

Also, compared to the New Forest and Dawes Farm, Friston Forest resides much closer to the sea shoreline, meaning the higher salt content in the area may potentially affect the salinity of the soil, relative to that of the other two locations; higher soil salinity has been shown to affect the microbial activity and metabolism of organisms within the soil, as well as changing the overall microbiome structure (Yan *et al*., 2015; Maganhotto de Souza Silva and Fay, 2012). In collaboration with the Forestry commission, several areas within Friston Forest were sampled to increase the likelihood of isolating pathogenic organisms, as well as APO that are vital in the fight against AMR.

4.1 Methods

4.1.1 Isolation and characterisation of microorganisms within the soil

The collection of soil samples from several areas within Friston Forest was carried out according to the methods stated in section 2.2.1, with coordinates and characteristics of each sample noted (see Appendix Table A3). Isolation and biochemical testing for the identification of potential pathogenic organisms within the soil was carried out using the methodology stated in 2.2.2.2 and 2.2.3.2. Sample drying, and isolation of APO and potential *Streptomyces* spp. was carried out using the methodology stated in 2.2.2.3, using both oat and potato starch agar, with further characterisation using Gram staining, and PCR for the identification of isolates belonging to the *Streptomyces* spp. carried out according to section 2.2.3.3.

4.1.2 Further testing of Antimicrobial Producing Organisms

All APO were further tested with the perpendicular screening method using both sensitive and clinical strains of test bacteria (see sections 2.2.4.2 and 2.2.4.3), as well as the starvation method using sensitive strains of *E. coli*, *S. epidermidis*, *S. aureus* and *P. mirabilis* (see section 2.2.4.5). Only repeats of novel antimicrobial using sensitive strains were carried out.

4.2 Results

Twenty soil samples were collected from Friston Forest, Sussex, at varying locations with differing characteristics in April 2017 (see appendix Table A3), for the cultivation of both APO and pathogenic organisms.

4.2.1 Isolation of pathogenic microorganisms

From the 20 soil samples collected (see appendix Table A3), 55 wet soil isolates were subcultured and purified ready for further testing and identification. The biochemical test results and morphology of each individual isolate can be seen in Table 4.1. No isolates from Friston Forest were identified as belonging to the *Klebsiella* spp. or identified as *E. coli*. Several isolated were seen to belong to the *Bacillus*, *Kurthia*, and *Acinetobacter* spp.; 13 (23.6%), eight (14.6%), and 12 (21.8%) respectively. *Kurthia* spp. was noted as above due to its irregular colony morphology resembling that of a 'birds feather'. Two isolates were identified as *S. aureus* with two identified as belonging to the *Salmonella* spp. (3.6%). One and two isolates were identified as *Enterococci* spp. (1.8%) and *Enterobacter* spp. (3.6%) respectively. Two isolates also had the presumptive identification as belonging to the *Streptomyces* spp. due to the test results (3.6%).

Table 4.1: The biochemical characteristics, colony morphology and presumed species of the 55 bacteria isolated from wet soil samples from Friston Forest. Morphology noted is the isolate as seen on nutrient agar. (+VE) indicates a positive result, and (-VE) indicates a negative result to the individual biochemical tests used; Catalase, Oxidase, Lactose fermentation, Mannitol fermentation, Edwards agar growth, Coagulase, Indole, Simmons' Citrate and Urease tests results are seen for the varying isolates. A lack of growth seen on the differing characterisation agar used, by isolates, is stated (No growth). Coagulase tests were carried out on Gram-positive cocci only. Blank results in the biochemical test sections, indicate that the specific test was not carried out on the corresponding isolate.

Isolate Number	Colony Size (mm)	Colony Morphology	Gram stain	Catalase	Oxidase	Lactose fermentation	Mannitol fermentation	Edwards agar growth	Coagulase test	Indole test	Citrate test.	Urease test.	Presumptive spp.
1.1	1	Small, White, circular, glossy.	Gram-positive rods.	+VE	+VE		No growth	No growth		-VE	-VE		*Rothia* spp.
1.2	3	Cream, circular, glossy.	Gram-negative rods	+VE	-VE	No growth				-VE		-VE	*Acinetobacter* spp.
1.3	7	Cream, circular with rough edges, glossy.	Gram-negative rods.	+VE	-VE	No growth				-VE		-VE	*Acinetobacter* spp.
1.4	3	Cream, circular glossy.	Gram-negative rods.	+VE	-VE	-VE					-VE	-VE	*Acinetobacter* spp.
2.1	2	White, Circular, glossy.	Gram-negative rods.	+VE	-VE	No growth					-VE	-VE	*Acinetobacter* spp.
2.2	4	White, irregular shape, glossy.	Gram-negative rods	+VE	-VE	No growth				-VE		-VE	*Acinetobacter* spp.
3.1	2	Cream, circular, glossy.	Gram-positive rods.	+VE	-VE		-VE	-VE		-VE	+VE		*Bacillus* spp.
3.2	1	White, circular glossy.	Gram-positive rods.	+VE	-VE		-VE	No growth		-VE	+VE		*Bacillus* spp.
4.1	1	White, clear, circular.	Gram-negative rods.	+VE	+VE	No growth				-VE	-VE	-VE	*Moraxella spp.*
5.1	7	Cream, circular with slightly rough edges, glossy.	Gram-negative rods.	+VE	-VE	No growth				-VE	-VE	-VE	*Acinetobacter* spp.
5.2	2	White, circular, matte.	Gram-positive rods.	+VE	-VE		No growth	No growth		-VE	-VE	-VE	*Kurthia* spp.
6.1	8	Cream, circular, glossy.	Gram-negative rods.	+VE	-VE	No Growth				-VE	-VE	-VE	*Acinetobacter* spp.

Isolate Number	Colony Size (mm)	Colony Morphology	Gram stain	Catalase	Oxidase	Lactose fermentation	Mannitol fermentation	Edwards agar growth	Coagulase test	Indole test	Citrate test.	Urease test.	Presumptive spp.
6.2	<1	White, circular, matte.	Gram-positive rods.	+VE	-VE	-VE	+VE			-VE	+VE		*Bacillus* spp.
6.3	1	Grey, circular, glossy.	Gram-negative rods.	+VE	+VE	No growth					-VE		*Moraxella* spp.
7.1	9	Cream, circular, matte.	Gram-positive rods.	+VE	-VE		-VE	No growth		-VE	+VE		*Bacillus* spp.
7.2	1	Cream, circular, glossy.	Gram-positive cocci.	+VE	-VE		-VE	No growth	+VE				*Staphylococcus aureus*.
7.3	3	Cream, circular, matte.	Gram-positive rods.	+VE	-VE		No growth	No growth		-VE	+VE		*Bacillus* spp.
7.4	1	White, glossy, circular.	Gram-positive rods.	-VE	+VE		No growth	No growth		-VE	-VE		*Actinomyces* spp.
8.1	6	Cream, circular, matte.	Long Gram-positive rods.	+VE	-VE		-VE	No growth		-VE	+VE		*Streptomyces* spp.
8.2	7	Cream, glossy.	Gram-negative rods.	+VE	-VE	-VE				-VE	+VE	-VE	*Salmonella* spp.
8.3	4	White, irregular shape, matte.	Gram-positive rods.	+VE	+VE		No growth			-VE	-VE		*Kurthia* spp.
9.1	5	Cream, circular, matte.	Gram-negative rods.	+VE	+VE.	-VE					-VE		*Moraxella* spp.
9.2	3	Yellow, circular, glossy.	Gram-positive cocci.	-VE	-VE		+VE	+VE					*Enterococci* spp.
9.3	4	White, matte, irregular shape.	Gram-positive rods.	+VE	+VE		-VE	No growth		-VE	-VE		*Rothia* spp.
9.4	1	Cream, circular, glossy.	Gram-positive rods with spores.	+VE	+VE		-VE	No growth		-VE	+VE		*Bacillus* spp.
9.5	5	White, circular, matte.	Gram-negative rods.	+VE	+VE	-VE				-VE	-VE		*Moraxella* spp.
11.1	11	White, irregular shape, matte.	Gram-positive rods.	+VE	-VE		No growth	No growth			-VE		*Kurthia* spp.
12.1	5	Cream, circular, matte.	Gram-positive rods.	+VE	-VE		-VE	No growth			+VE		*Bacillus* spp.
12.2	1	Grey, circular, glossy.	Gram-negative rods	+VE	-VE	+VE				-VE	+VE	-VE	*Enterobacter* spp..

Isolate Number	Colony Size (mm)	Colony Morphology	Gram stain	Catalase	Oxidase	Lactose fermentation	Mannitol fermentation	Edwards agar growth	Coagulase test	Indole test	Citrate test.	Urease test.	Presumptive spp.
12.3	6	White, matte, circular.	Gram-positive rods.	+VE	-VE		No growth	No growth		-VE	-VE		*Kurthia* spp.
13.1	7	Cream, circular, matte.	Gram-negative rods.	+VE	-VE	No growth					-VE		*Streptomyces* spp.
13.3	6	White, irregular shape, matte.	Gram-positive rods.	+VE	+VE		No growth	No growth		-VE	-VE		*Kurthia* spp.
14.1	7	Cream, matte, circular.	Gram-negative rods.	+VE	-VE	No growth				-VE	+VE	-VE	*Acinetobacter* spp.
14.2	6	Cream, circular, matte.	Gram-negative rods.	+VE	-VE	No growth				-VE	+VE	-VE	*Acinetobacter* spp.
14.3	2	White, glossy, circular.	Gram-negative rods.	+VE	-VE	+VE				-VE	-VE	-VE	Erwinia spp.
14.4	<1	Grey, circular, glossy.	Gram-negative rods.	+VE	-VE	No growth						-VE	*Acinetobacter* spp.
14.5	1	Grey, circular, glossy.	Gram-negative rods.	+VE	-VE	+VE				-VE	+VE (blue agar).	-VE	*Enterobacter* spp.
15.2	4	White, irregular shape.	Gram-negative rods.	+VE	+VE	No growth				-VE	-VE		*Moraxella* spp.
16.1	2	Cream, matte, circular.	Gram-negative rods.	+VE	-VE	No growth				-VE	+VE	-VE	*Acinetobacter* spp.
16.2	3	Cream, matte, circular.	Gram-positive rods.	+VE	+VE		+VE	No growth		-VE	+VE		*Bacillus* spp.
16.3	3	Cream, circular, glossy.	Gram-negative rods.	+VE	-VE	No growth				-VE	-VE	-VE	*Cedecea* spp.
17.1	13	Grey, matte, circular.	Gram-positive rods.	+VE	+VE		+VE	No growth		-VE	-VE		*Kurthia* spp.
17.2	6	Cream, circular, matte.	Gram-negative rods.	+VE	-VE	No growth.	No growth.					-VE	*Acinetobacter* spp.
17.3	<1	White, circular, glossy.	Gram-positive cocci.	+VE	-VE		-VE	No growth	+VE				*Staphylococcus aureus*.
17.4	1	Grey, circular, glossy.	Gram-negative rods.	+VE	+VE	No growth				-VE	-VE		*Moraxella* spp.
18.1	4	Cream, circular, matte.	Gram-positive rods.	+VE	-VE		-VE	No growth		-VE	+VE		*Bacillus* spp.

Isolate Number	Colony Size (mm)	Colony Morphology	Gram stain	Catalase	Oxidase	Lactose fermentation	Mannitol fermentation	Edwards agar growth	Coagulase test	Indole test	Citrate test.	Urease test.	Presumptive spp.
18.2	49	White, irregular shape, matte.	Gram-positive rods.	+VE	+VE		No growth	No growth		-VE	-VE		*Kurthia* spp.
18.3	<1	White, irregular shape, matte.	Gram-positive rods.	+VE	+VE		No Growth	No growth		-VE	-VE		*Kurthia* spp.
19.2	2	Cream, circular, glossy.	Gram-negative rods.	+VE	-VE	-VE				-VE	+VE	-VE	*Salmonella* spp.
19.3	9	White, irregular shape, matte.	Gram-positive rods with spores.	+VE	+VE		+VE	No growth		-VE	+VE	-VE	*Bacillus* spp.
19.4	12	White, irregular shape, matte.	Gram-positive rods with spores.	+VE	+VE	-VE	No growth				+VE		Bacillus spp.
20.1	5	Cream, circular, matte.	Gram-positive rods.	+VE	-VE		-VE	No growth		-VE	+VE	-VE	*Bacillus* spp.
20.2	7	White, circular with rough edges, matte.	Gram-positive rods with spores.	+VE	+VE		+VE				+VE		*Bacillus* spp.
20.3	2	White, circular, glossy.	Gram-negative rods	+VE	+VE	-VE				-VE	+VE		*Pseudomonas* spp.
20.4	1	White, circular, matte.	Gram-negative rods.	+VE	+VE	No growth				-VE	-VE		*Moraxella* spp.

4.2.2 Identification of *Streptomyces* spp. from APO isolated

Using the dried soil samples from the 20 samples sites, a total of 69 APO with either displayed inhibition and/or similar morphology to that of the *Streptomyces* genus, were isolated using both oat agar (31 isolates) and potato starch agar (38 isolates), before being Gram stained and catalase tested (see appendix Tables A4 and A5). Out of these isolates cultivated from Friston Forest, 33 isolates were noted to have similar morphology and/or Gram stain to that of the *Streptomyces* spp.; 13 and 20 isolates cultivated using oat and potato starch agar respectively, were further tested using PCR for the identification of those belonging to the *Streptomyces* genus (Table A6 and A7). Seven APO isolated using oat agar was found to presents bands at ~1500bp – indicating they belong to the *Streptomyces* spp. (22.6%) (Table A6). Totally 12 (31.6%) out of the 38 isolates obtained using potato starch agar were found to belong to the *Streptomyces* spp. (Table A7). Figure 4.1 details a gel containing PCR reaction mixtures of APO 68, 75, 81, 84, 88, and 91, separated using gel electrophoresis. From this gel isolates 81, 84 and 91 were confirmed as belonging to *Streptomyces* spp., with isolate 88 determined as belonging to another genus. PCR was repeated for those displaying only one band of the two duplicates; 68 and 75 were seen to produce only one band at 1500bp, out of the two duplicates (Figure 4.1).

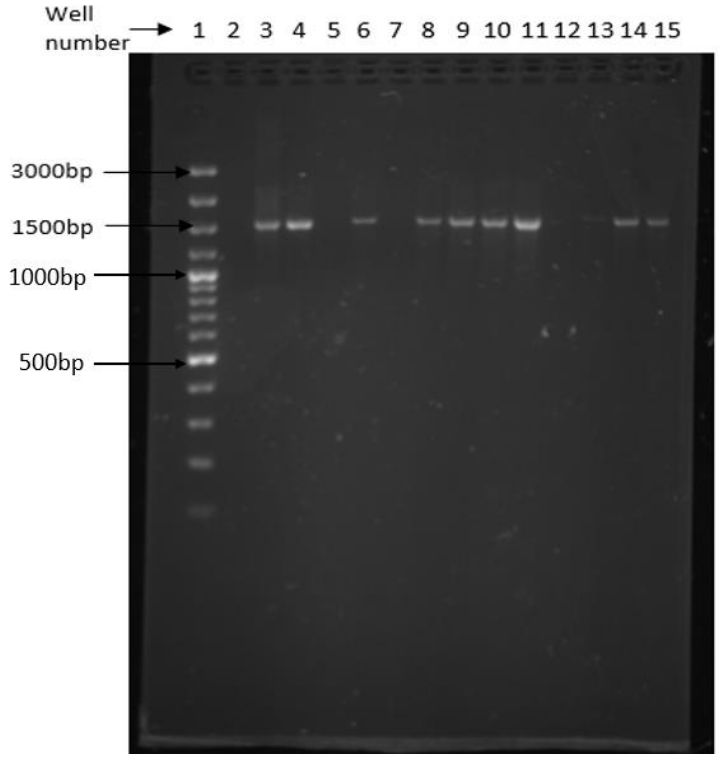

Figure 4.1: Agarose gel (2%) run of PCR reaction mixtures using DNA extracted from Friston Forest Isolates, cultivated using potato starch agar, for the identification of *Streptomyces* spp. Bands were visualised at ~1500bp, indicating the amplification of the 16S rRNA gene within the *Streptomyces* spp. Wells were filled with PCR reaction mixtures as follows: 1= DNA ladder, 2= No template DNA, 3= *S. coelicolor* (positive control), 4= isolate 68, 5= isolate 68, 6= isolate 75, 7= isolate 75, 8= isolate 81, 9 = isolate 81, 10 = isolate 84, 11= isolate 84, 12= isolate 88, 13= isolate 88, 14= isolate 91, 15= isolate 91.

4.2.3 Testing of Antimicrobial Producing Organisms

4.2.3.1 Novel antimicrobial screening against sensitive organisms

All isolates yielded from dry soil screening using oat and potato starch agar (31 and 38 microorganisms respectively), due to inhibitory properties and/or similarities to *Streptomyces* spp. morphology, were initially tested using the perpendicular screening method.

Initial screening detailed seven (21.2%) out of the 31 oat agar isolates with antimicrobial activity, with isolate 60 displaying inhibitory activity against *P. mirabilis*, and other isolates displaying inhibition against *E. coli, S. epidermidis,* and/or *S. aureus* (Table 4.2). Four out of the 38 (10.5%) organisms isolated using potato starch agar for initial cultivation, displayed inhibitory activity, with inhibition being seen only against *E. coli*, *S. epidermidis* and *S. aureus* (Table 4.4). Figure 4.2 displays the inhibition seen towards *P. mirabilis* by isolate 60, after 2 days incubation prior to the addition of test bacteria, at 25°C. Figures 4.4 and 4.5 show the activity of isolates 68 and 70, displaying inhibition after 2 days incubation at 25°C during initial perpendicular screening; 68 displayed inhibition towards *S. epidermidis* and *S. aureus*, and 70 displayed inhibition against *S.*

epidermidis, *S. aureus* and *E. coli*. In total 15.9% of all isolates, cultivated using both agars, from Friston Forest were seen to display inhibition.

Repeats of perpendicular screening of all isolates, with previously identified inhibition, however detailed less and differing inhibition to initial screening results (Tables 4.3 and 4.5 respectively). Four out of the 7 (57.1%) oat isolates displayed continued inhibition of some form, and two out of the four (50%) potato starch agar isolates, again showed inhibition. Out of the 69 Friston isolates, 11 displayed inhibitory activity against one of the test organisms (15.9%), with six displaying repeat inhibitory activity (8.7%). Figure 4.3 displays the inhibition against *E. coli*, *S. epidermidis* and *S. aureus* after 2 days incubation at 25°C of oat isolate 64, in the repeat of perpendicular screening.

Table 4.2: The seven APO isolated from Friston Forest using oat agar as initial cultivation media, that displayed inhibitory activity in initial perpendicular screening. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Inhibition of test bacteria seen at 25°C**		**Inhibition of test bacteria seen at 37°C**	
	0 day	**2 day**	**0 day**	**2 day**
38	-	*E. coli, S epidermidis* and *S. aureus*	-	-
49	-	*E. coli, S. aureus* and *S. epidermidis*	-	-
53	-	-	-	*S. epidermidis*.
54	-	*S. aureus*.	-	-
57	-	-	*S. aureus*.	*S. epidermidis* and *S. aureus.*
60	-	*P. mirabilis*	-	-
64	-	*E. coli*.	-	-

Table 4.3: Inhibition results from the repeat of the perpendicular screening method, of the seven Friston Forest APO isolated using oat agar, that showed inhibition previously. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
38	-	-	-	-
49	-	*S. epidermidis*	-	-
53	-	-	-	-
54	-	-	*S. epidermidis* and *S. aureus*	-
57	-	-	-	-
60	-	*P. mirabilis*	-	*P. mirabilis*
64	-	*E. coli, S. epidermidis and S. aureus*	-	*S. aureus*

Table 4.4: The four isolates from Friston Forest, cultivated using potato starch agar displaying inhibitory activity from initial screening using the perpendicular screening method. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
68	-	*S. epidermidis* and *S. aureus*.	-	-
70	*S. epidermidis* and *S. aureus*.	*E. coli, S. epidermidis* and *S. aureus*.	*E. coli*.	-
100	-	*S. epidermidis*.	-	-
101	-	*E. coli, S. epidermidis* and *S. aureus*.	-	-

Table 4.5: The inhibition seen in the repeat of perpendicular screening, of the four Friston Forest APO, cultivated using potato starch agar, that had previously shown inhibition. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
68	-	*S. epidermidis* and *S. aureus*.	-	-
70	-	-	-	-
100	-	-	-	-
101	-	*E. coli, S. epidermidis* and *S. aureus*.	-	-

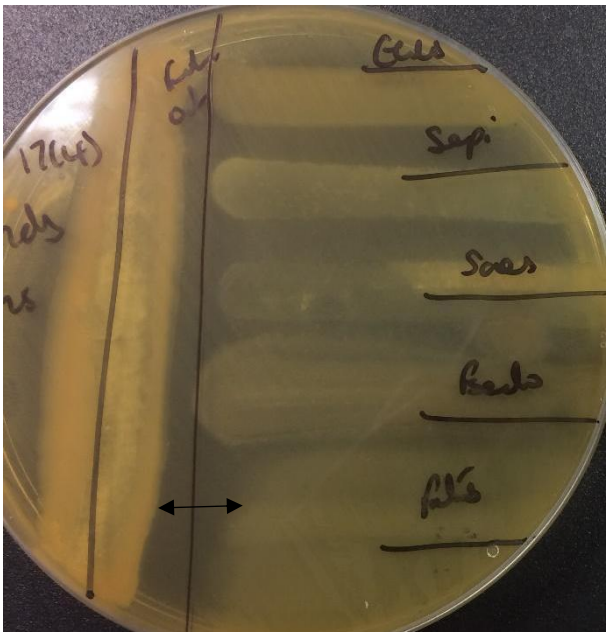

Figure 4.2: The inhibition seen by Friston Forest isolate 60, isolated using oat agar, against *P. mirabilis*, after 2 days incubation at 25°C. The arrow indicates where the growth of *P. mirabilis* has been inhibited. From top to bottom, *E. coli*, *S. epidermidis*, *S. aureus*, *P. aeruginosa* and *P. mirabilis* were tested against isolate 60.

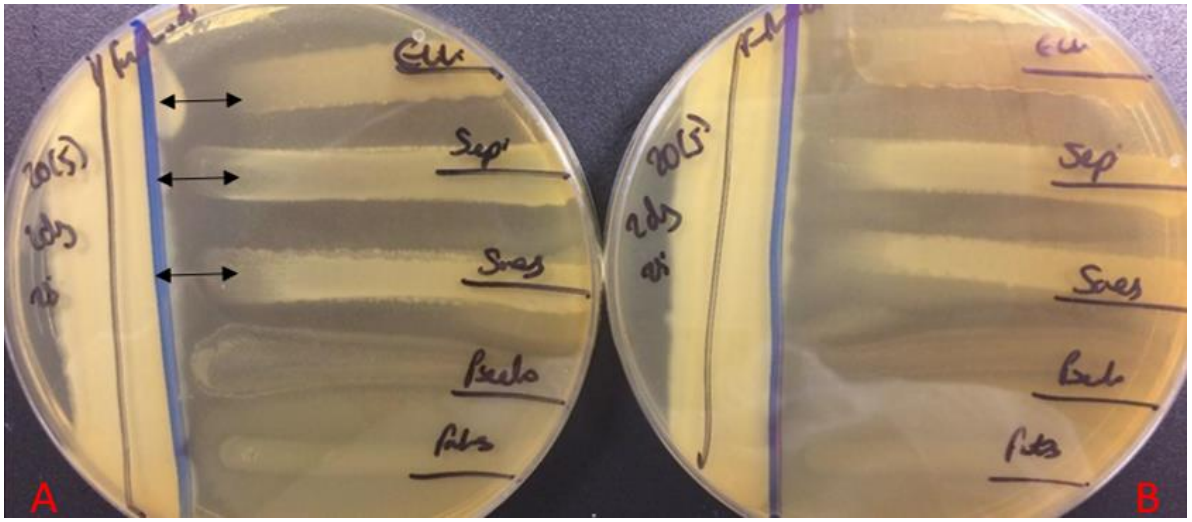

Figure 4.3: The inhibitory action of Friston oat isolate 64 in duplicate (A and B), against *E. coli*, *S. epidermidis*, and *S. aureus*, after incubation for 2 days at 25°C, upon the repetition of the perpendicular screening method. The arrows indicate where the individual organisms' growth have been inhibited. From top to bottom, *E. coli*, *S. epidermidis*, *S. aureus*, *P. aeruginosa* and *P. mirabilis* were tested against isolate 64.

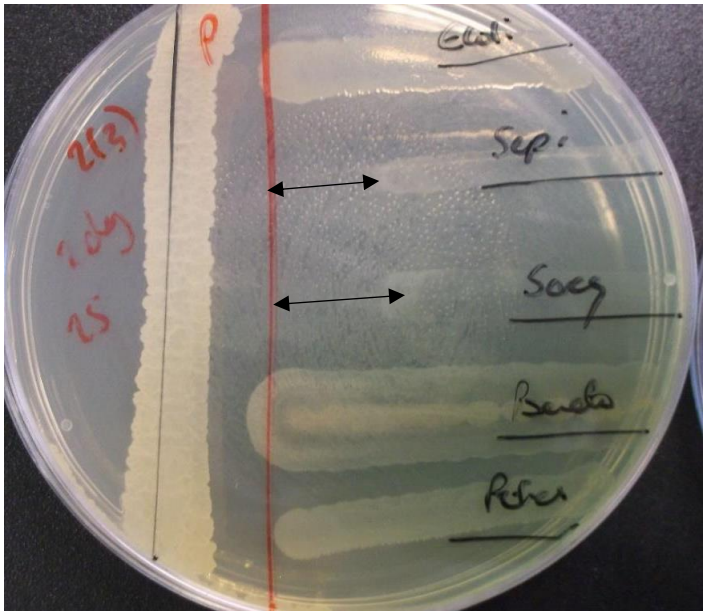

Figure 4.4: Friston Forest isolate 68 cultivated using potato starch agar, displaying inhibition against *S. epidermidis* and *S. aureus* after 2 days incubation at 25°C before the addition of the test bacteria in the initial perpendicular screening method. The black arrows indicate the extent of the inhibition of test bacteria caused by APO 68. From top to bottom, *E. coli*, *S. epidermidis*, *S. aureus*, *P. aeruginosa* and *P. mirabilis* were tested against isolate 68.

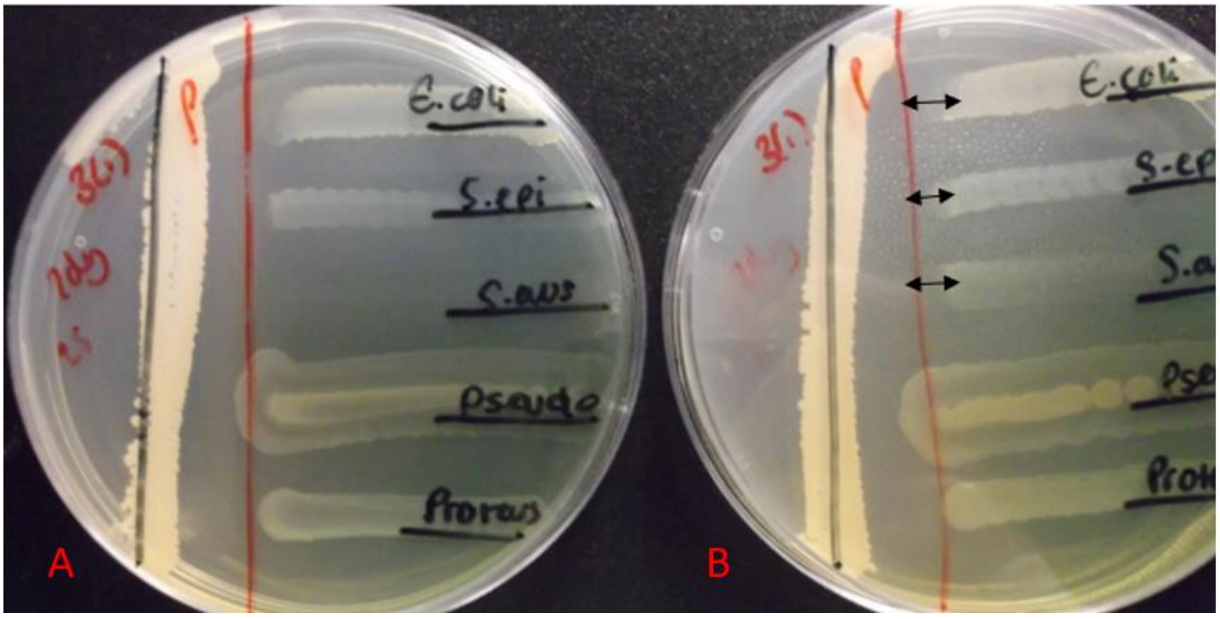

Figure 4.5: Isolate 70 initially cultivated from Friston Forest using potato starch agar, displaying inhibition against *E. coli*, *S. epidermidis* and *S. aureus* after 2 days incubation at 25°C, in duplicate (A and B), in initial perpendicular screening. From top to bottom, *E. coli*, *S. epidermidis*, *S. aureus*, *P. aeruginosa* and *P. mirabilis* were tested against isolate 70.

4.2.3.2 Antimicrobial screening against clinical strains

The six APO originally isolated from Friston Forest using oat agar, that showed inhibition towards sensitive strains of *E. coli, P. mirabilis* and/or *S. aureus,* were further tested against clinically isolated strains of the same species of bacteria, using the perpendicular streak method. Table 4.6 displays that two APO showed further inhibition towards the clinical strains of test bacteria (33.3%). Isolate 53 previously only displayed inhibition to *S. epidermidis*, and hence was not further tested against any of the clinical isolates (Table 4.2). Figure 4.6 highlights inhibition presented towards the clinical strain of *P. mirabilis*, by APO 60, after it was incubated for 2 days at 25°C prior to the addition of test bacteria.

Three of the original four APO isolated from Friston Forest using potato starch agar with inhibition seen previously, were further tested using clinical strains; isolate 100 was not further tested due to inhibition only being seen against *S. epidermidis* (Table 4.4). Only one of the three potato isolates displayed inhibition to the clinical strains of test bacteria (33.3%) (Table 4.7). Inhibition seen towards the clinical strains of *E. coli* and *S. aureus* by Friston isolate 101, can be seen in Figure 4.7, where the organism was grown for 2 days at 25°C prior to the addition of test bacteria.

Table 4.6: Inhibition seen towards clinical strains of test bacteria by Friston Forest APO isolated using oat agar, after both 0 and 2 days incubation at both 25°C and 37°C, using the perpendicular screening method. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Test Organisms**	**Inhibition of test bacteria seen at 25°C**		**Inhibition of test bacteria seen at 37°C**	
		0 day	**2 day**	**0 day**	**2 day**
38	*E. coli*, and *S. aureus*.	-	-	-	-
49	*E. coli*, and *S. aureus*	-	-	-	-
54	*S. aureus*.	-	-	-	-
57	*S. aureus*.	-	-	-	-
60	*P. mirabilis*.	-	*P. mirabilis*.	-	*P. mirabilis*.
64	*E. coli*, and *S. aureus*	-	*E. coli* and *S. aureus*.	-	-

Table 4.7: Inhibition seen towards clinical strains of test bacteria by APO isolated from Friston Forest using potato starch agar, incubated for both 0 and 2 days at both 25°C and 37°C, using the perpendicular screening method. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Test Organisms**	**Inhibition of test bacteria seen at 25°C**		**Inhibition of test bacteria seen at 37°C**	
		0 day	**2 day**	**0 day**	**2 day**
68	*S. aureus*.	-	-	-	-
70	*S. aureus and E. coli*.	-	-	-	-
101	*S. aureus and E. coli*.	-	*E. coli* and *S. aureus*.	-	-

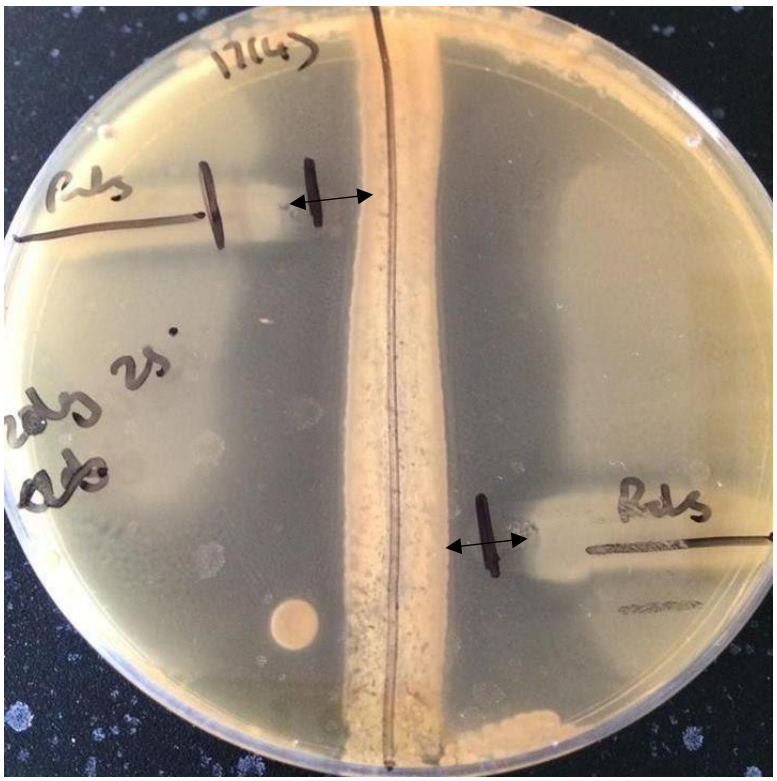

Figure 4.6: The inhibition towards the clinical strain of *P. mirabilis*, presented by Friston oat isolate 60, that was incubated for two days at 25°C before the addition of test bacteria. The arrows indicate the extent of the inhibition of *P. mirabilis* caused by isolate 60.

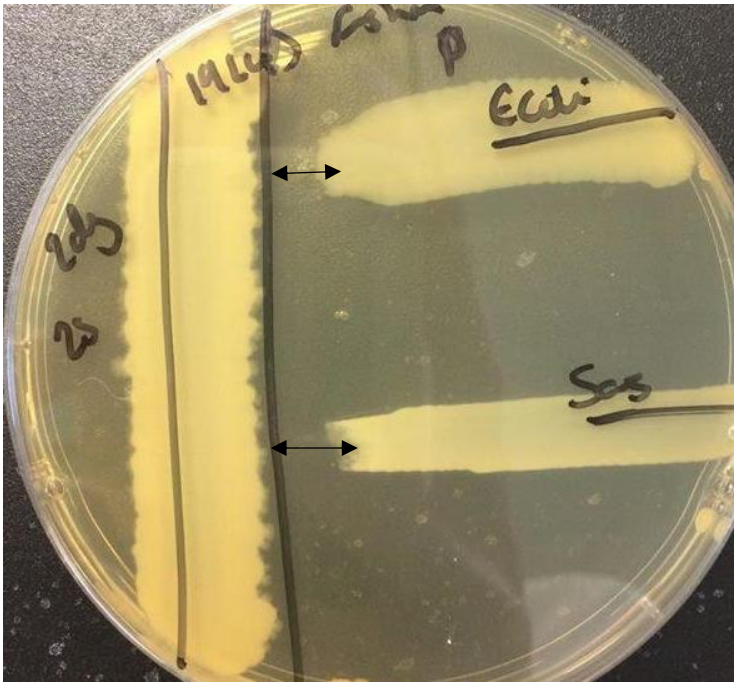

Figure 4.7: The inhibition presented by Friston isolate 101, originally isolated using potato starch agar, towards resistant clinical strains of *E. coli* (top) and *S. aureus* (bottom) from the perpendicular screening method. Isolate 101 was incubated for 2 days at 25°C before the addition of test bacteria and further incubated for 7 days. The arrows indicate the inhibitory activity by isolate 101 towards the test organisms.

4.2.3.3 Starvation method

Isolates cultivated from Friston Forest using both oat and potato starch agar, were tested using the starvation method. Five of the 31 (16.1%) oat isolates displayed inhibition of at least one of the test bacteria used at the differing temperatures and timepoints (Table 4.8). Isolate 53 showed inhibition at a range of time points, as well as inhibition at both 25°C and 37°C. Figure 4.9 displays an example of the inhibitory activity produced by isolate 53 towards *S. epidermidis*, after it was incubated for 10 days at 25°C. Figure 4.8 also shows the inhibition seen towards *S. epidermidis* on Mueller-Hinton agar, by oat isolate 60 that was incubated for 14 days at 37°C.

Out of the 38 APO isolates from potato starch agar, 16 (42.1%) showed some form of inhibition towards the test bacteria used (Table 4.9). Isolate 78 was seen to display inhibition against *S. aureus*, streaked as a lawn on plate count agar, after incubation for 7 days at 37°C (figure 4.10). Figure 4.11 displays the inhibition presented towards *E. coli* on Mueller-Hinton agar, after isolate 98 was incubated for 10 days at 37°C. Repeats of this starvation method, using stored supernatant, as well as repeat of whole method, did not occur for all Friston Forest isolates, due to time constraints.

In total the number of Friston Forest APO, cultivated using both agars, that displayed inhibition towards one of the test bacteria in the starvation method, was 30.4%, with a large

majority inhibiting the Gram-positive test organisms used. Ten out of the 16 (62.5%), and three out of the five (60%) confirmed APO, isolated using potato and oat agar respectively, were able to inhibit at least one of the test *Staphylococcus* spp. used.

Table 4.8: The inhibition seen by APO isolated from Friston Forest using oat agar, tested using the starvation method. The agar that inhibition was seen on by individual isolates, is stated in brackets; PC – plate count agar, MH- Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number	**Days of incubation of isolate at 25°C (agar result in bracket)**			**Days of incubation of isolate at 37°C (agar result in bracket)**		
	7 days	**10 days**	**14 days**	**7 days**	**10 days**	**14 days**
47	-	-	-	*E. coli* (PC).	*S. aureus* (PC) *E. coli* (MH).	-
53	*S. aureus* (PC).	*S. aureus* and *S. epidermidis* (PC).	*S. epidermidis* and *S. aureus* (PC).	-	-	*S. aureus* (PC).
54	-	-	-	-	*E. coli* (PC).	-
60	-	-	-	*E. coli*, *S. aureus* and *S. epidermidis* (MH).	*E. coli*, *S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and *S. aureus* (PC and MH) and *E. coli* (MH).
63	-	-	-	-	*E. coli* (MH)	-

Table 4.9: The inhibition seen by the APO isolated from Friston Forest using potato starch agar, using the starvation method. The agar that inhibition was seen on by individual isolates, is stated in brackets; PC – plate count agar, MH - Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	**Days of incubation at 25°C (agar result in bracket)**			**Days of incubation at 37°C (agar result in bracket)**		
	7 days	**10 days**	**14 days**	**7 days**	**10 days**	**14 days**
69	-	-	-	*E. coli* (MH + PC).	-	-
70	*S. epidermidis* and *S. aureus* (PC).	-	-	*S. epidermidis* and *S. aureus* (PC) *E. coli* (MH + PC).	-	*E. coli* (PC + MH).
71	-	-	-	*E. coli* (MH + PC).	-	-
73	-	*S. aureus* and *S. epidermidis* (PC)	-	-	*E. coli* (MH).	*S. epidermidis* and *S. aureus* (MH).
74	-	*S. epidermidis* and *S. aureus* (MH + PC).	-	-	-	-
75	-	-	-	-	*E. coli* (MH)	*E. coli* (MH) and *S. epidermidis* (PC).
76	-	*S. epidermidis* and *S. aureus* (PC + MH)	-	-	-	-
78	*S. epidermidis* and *S. aureus* (PC).	-	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and *S. aureus* (PC).	*S. epidermidis* and *S. aureus* (PC).
82	-	-	-	*E. coli* (MH).	-	-
84	-	-	-	*E. coli* (PC).	*E. coli* (MH).	*E. coli* (MH + PC).
85	**-**	**-**	**-**	**-**	**-**	*E. coli, S. epidermidis* and *S. aureus* (MH).
90	-	-	*S. aureus* (MH+PC) and *S. epidermidis* (PC).	-	-	-
96	-	*S. epidermidis* and *S. aureus* (PC)	*S. aureus* (PC).	-	-	-
97	-	-	-	-	-	*E. coli* (MH)
98	-	-	-	-	*E. coli* (MH).	-
102	-	-	*S. epidermidis* and *S. aureus* (PC).	-	-	-

Figure 4.8: Isolate 60 Isolated from Friston Forest using oat agar, incubated for 14 days at 37°C, detailing inhibition against *S. epidermidis* streaked as a lawn on Mueller-Hinton agar, using the starvation method. The arrow provides an example as well as indicating the inhibition of *S. epidermidis* caused by isolate 60.

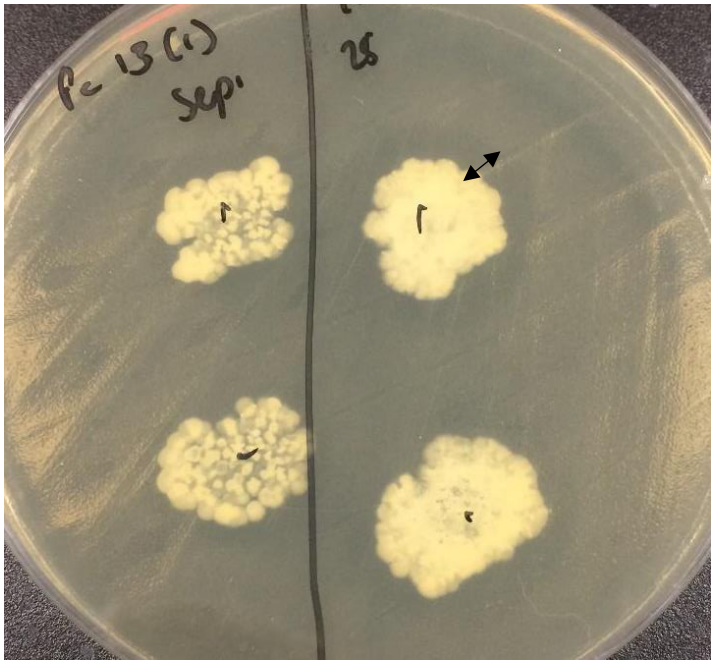

Figure 4.9: The inhibition seen towards *S. epidermidis* on plate count agar, by Friston Forest oat isolate 53, that was incubated for 10 days at 25°C, using the starvation method. The arrow indicates an area of inhibition of *S. epidermidis* caused by isolate 53.

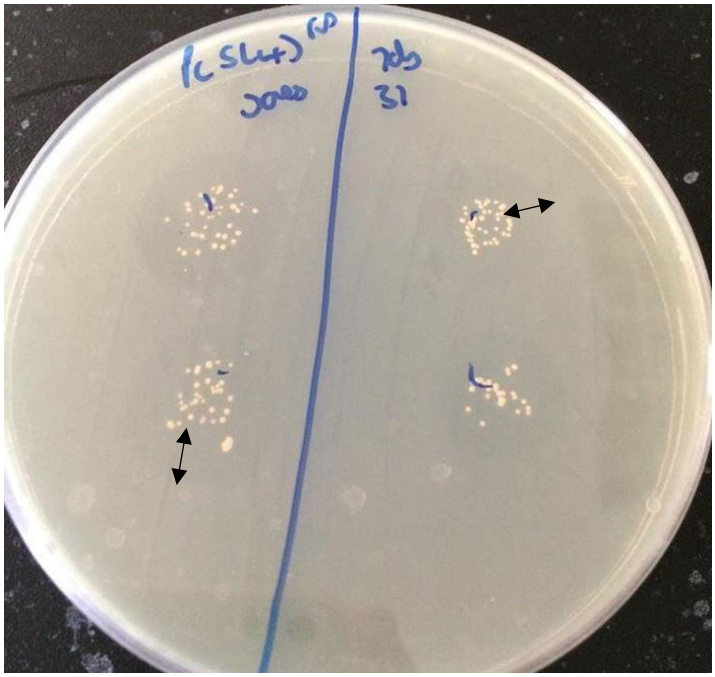

Figure 4.10: Isolate 78, initially isolated from Friston Forest using potato starch agar, displaying inhibition against *S. aureus*, on plate count agar, after 7 days incubation at 37°C, using the starvation method. An example of the extent of the inhibition towards *S. aureus* can be seen by the arrows used.

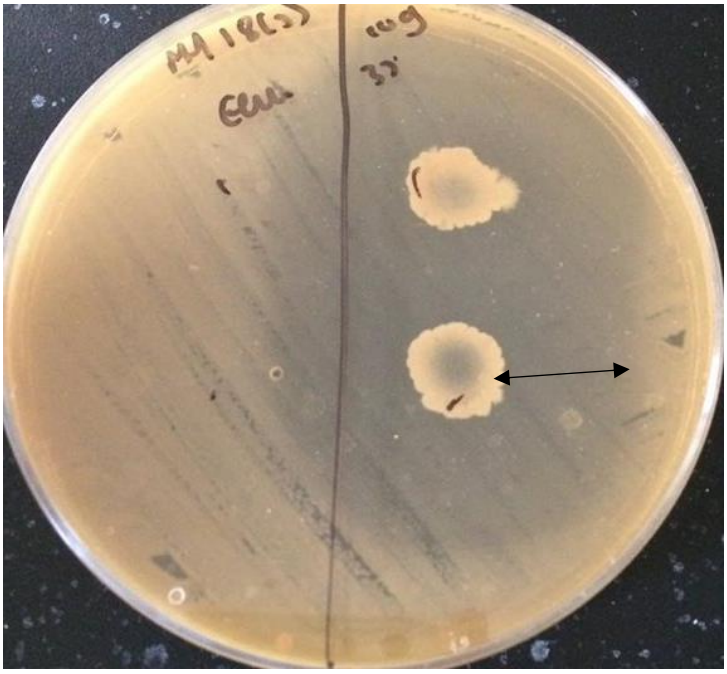

Figure 4.11: Isolate 98 isolated from Friston Forest using potato starch agar, displaying inhibition towards *E. coli* on Mueller-Hinton agar, in the starvation method, after 10 days incubation at 37°C. The arrow indicates the inhibition of *E. coli* seen, caused by the inhibitory activity of isolate 98.

4.3 Discussion

Friston Forest was selected due to its classification of being a modern forest, allowing the results obtained to be compared to that of New Forest, as well as Dawes Farm. From these results, it can be seen that APO, can in fact be cultivated from Friston Forest, with 69 organisms initially cultivated due to observed inhibition and/or similar morphology to that of *Streptomyces* spp. Using oat agar, 31 APO were initially selected and purified, before testing using perpendicular screening. Using this method, it was detailed that seven (21.2%) of these had the ability to produce some form of inhibitory action (Table 4.2), including isolate 60 that displayed inhibition against *P. mirabilis* after 2-day incubation at 25°C (figure 4.2), with the six other APO showing inhibition towards either *E. coli*, *S. epidermidis* and/or *S. aureus*. Upon repeating this method, isolate 60 again showed inhibition towards *Proteus* spp. after 2-day incubation, but this inhibition was seen at both 25°C and 37°C, showing increase in inhibitory activity (Table 4.3). As well as this isolate 64 also displayed further inhibition to both the *Staphyloccocus* spp., additional to the inhibition seen towards *E. coli*, in the first run of the method, however from this repeat, four of the APO showed less/no inhibition compared to the first run of the method (Tables 4.2 and 4.3). This lack of inhibition was also seen in the repeat of this method using the clinical test organisms (Table 4.6). Of the six isolates that displayed inhibition to either *E. coli*, *S. aureus* or *P. mirabilis* previously, only two (33.3%) displayed the ability to inhibit the clinical strains of the same test organism; isolates 60 and 64 displayed inhibition towards *P. mirabilis* from Brighton hospital, and *E. coli* and the NCTC strain of MRSA respectively. Although, the lack of inhibition seen may be due to the resistance possessed by both the *E. coli*, and MRSA, meaning the compounds being produced by the APO are ineffective.

Similar results were also seen by the Friston APO cultivated using potato starch agar, with less inhibition seen in both repeats of perpendicular screening. Overall 38 APO were isolated using this agar, with only four (10.5%), showing some form of inhibitory activity (Table 4.4). This shows that just over half the amount of inhibition was seen compared to the oat agar isolates (21.2%), with no inhibition seen towards *P. mirabilis*. The potato starch agar isolates also displayed less inhibition upon the first repeat, with only two of the four isolates displaying inhibition; isolates 68 and 101 continued to produce inhibitory compounds (Table 4.5). Upon the further testing using clinical organisms, only one of the three APO tested (33.3%), displayed inhibitory activity; figure 4.7 shows the inhibition of clinical strains of both *E. coli* and MRSA, by isolate 101. However, these results still show that APO with inhibitory activity against clinical strains, are able to be isolated from Friston Forest, with inhibition also seen towards these bacteria by 33.3% of the oat agar isolates tested (Tables 4.6 and 4.7). As well as this both isolates 64, and 101, as discussed previously, were seen to produce compounds able to inhibit both resistant Gram-positive and Gram-negative organisms, displaying that further work on these compounds, including identification of its base structure using DOSY-NMR, could allow the production of a novel antimicrobial, able to treat these

potentially life-threatening infections in a clinical setting. However, neither of the isolates are seen to display inhibition towards, *P. aeruginosa*, a well-known opportunistic pathogen, and cause of urinary tract infections, and ventilator-associated pneumonia (Mittal *et al*., 2009; Sawa *et al*., 2016).

Further inhibition was also seen by potato starch agar isolates in the starvation method, with 16 (42.1%) isolates displaying some form of inhibition, much more than seen previously in the perpendicular screening method (10.5%) (Tables 4.4 and 4.9). This, however was not seen by the isolates cultivated using oat agar, with only five isolates (16.1%) showing some form of inhibitory activity in the starvation method, compared to the seven (21.2%) inhibitory isolates seen prior (Tables 4.2 and 4.8). This may provide further evidence for the specific conditions required, to encourage differing organisms to produce antimicrobial compounds, and it may be noted that these stress-induced conditions may, in fact, halt the production of the antimicrobial metabolites, and the lack of nutrients may have even caused cell death (Rokem *et al*., 2007). Although this shows a decrease of inhibitory activity from this method, some of the APO that did not display any inhibition in the initial perpendicular screening, were seen to inhibit test organisms in the starvation method; this was seen by oat isolates 47 and 63, which displayed a lack of inhibition in initial screening, but inhibited *E. coli* and *S. aureus*, and *E. coli* only respectively, after prolonged incubation (Tables 4.2 and 4.8). However, overall from this starvation method, 21 (30.4%) of the 69 APO were seen to produce some form of inhibition, compared to the 11 (15.9%) isolates in initial screening, showing an overall increase in inhibitory activity of the Friston Forest APO. Using this starvation method, it was also concluded that the majority of the inhibition was seen against the Gram-positive organisms, with three out of five oat isolates (60%) and 10 out of 16 (62.5%) potato starch agar isolates displaying activity against these test organisms (Table 4.8 and 4.9).

As well as this, certain APO also showed the ability to produce compounds under a range of conditions in the starvation method, including inhibition of test bacteria after differing lengths of incubations, as well as at both of the test temperatures used. Isolates 70 and 73 displayed the ability to produce inhibitory compounds after several of the different days incubations used, at both 25°C and 37°C, affecting both Gram-positive and Gram-negative bacteria (Table 4.9). This was also seen by isolates 53 and 78, which, under a range of conditions showed the ability to inhibit Gram-positive organisms (Tables 4.8 and 4.9). However, the repeat of this method using samples from both the same and differing locations within Friston Forest, may increase the chances of isolating APO, as well as allowing further conclusions to be drawn regarding the effectiveness of the starvation method.

From comparison of all APO results, certain organisms can be determined as highly effective at producing antimicrobial compounds. Such APO as isolates 53, 60 and 70, which all displayed inhibition in both initial perpendicular screening and starvation method, with isolate 60

also inhibiting the clinical strain of *P. mirabilis*, could be selected for further work including analysis of the compounds being produced, in the hope of isolating a novel antimicrobial compound (Tables 4.3, 4.4, 4.6, 4.8 and 4.9).

As well as the further testing of the APO, PCR was undertaken for the identification of those belonging to the *Streptomyces* genus, from both the oat and potato starch agar isolates with similar morphology, and Gram stain to that known of the species. In total 19 (27.5%) of the 69 Friston APO isolated were confirmed as belonging to the *Streptomyces* spp. with eight (42.1%) of these displaying some form of inhibitory activity in one or more of the screening methods used. However, as well as this an additional ten (14.5%) isolates could only be presumed as belonging to this genus, due to time constraints preventing the testing of these isolates using the PCR method. This highlights a key area of further research necessary, meaning conclusions regarding the presence and likelihood of isolating this genus at certain sample areas can be drawn, as well as allowing comparison and optimisation of the culture methods, to increase the likelihood of obtaining larger amounts of organisms belonging to the *Streptomyces* genus.

Of the 19 confirmed *Streptomyces* spp. varying inhibition was seen. Isolate 49 produced inhibitory activity towards *E. coli*, *S. aureus* and *S. epidermidis* in initial perpendicular screening, and *S. epidermidis* in the repeat of this method (Tables 4.2 and 4.3). However, upon testing of activity against clinical isolates, no inhibition was presented, although a strain of *S. epidermidis* was not available for use in this method, meaning future inclusion of this strain may produce differing results (Table 4.6). This is similar to isolates 53 and 100, which displayed inhibition towards *S. epidermidis* in the first repeat of perpendicular screening, however it is not known if the activity would inhibit the growth of a clinical strain of the same organism (Tables 4.2 and 4.4). Isolate 53 as well as 85 also detailed activity in the starvation method, however both these isolates were not confirmed as belonging to this genus due to the halting of the PCR procedure (Table 4.8 and 4.9). This can also be said for the presumed *Streptomyces* isolate 60 which displayed a broad range of activity, as discussed above (Tables 4.2, 4.3, 4.6 and 4.8). Other presumed *Streptomyces* spp. were seen to produce inhibition in the initial perpendicular screening against both clinical and sensitive strains of test bacteria including isolate 64 (Table 4.6), meaning further work, as discussed previously, using the PCR method to confirm the genus of these isolates, would provide further evidence for the inhibitory ability of compounds produced by the *Streptomyces* spp. (Gebreyohannes *et al.,* 2013).

Similar to the isolates cultivated using oat agar, from the same samples sites, none of the 12 confirmed *Streptomyces* spp., isolated using potato starch agar, showed inhibition spanning the differing methods, even though higher amounts of *Streptomyces* spp. were isolated using this agar (Table A7). Isolate 100 displayed inhibition in perpendicular screening as discussed above, and

isolates 76, 84, 96, 97, 98 and 102 were all active in the starvation method alone (Tables 4.4 and 4.9). As well as this, inhibition was also seen by isolate 85 in the starvation method, however this isolate was only presumed to belong to the *Streptomyces* genus, due to its characteristics, as discussed previously (Tables 4.9 and A5).

From the same 20 soil samples, 55 organisms were isolated and characterised using the several biochemical tests, for the identification of potential pathogenic bacteria, including those previously selected as of importance (see section 2.2.2.2). Two isolates (3.6%) were identified as belonging to the *Salmonella* spp., one of the genus' selected as of importance. A further two isolates (3.6%) were identified as *S. aureus* and belonging to the *Enterobacter* spp., as well as *Streptomyce*s spp. Additionally, the more well-known soil microorganisms such as *Kurthia* spp. (14.6%), *Bacillus* spp. (23.6%) and *Acinetobacter* spp. (21.8%) were isolated. These results hence may display the lack of potentially pathogenic organisms within the Friston Forest area. On the other hand, the lack of cultivation of these organisms may be due to the soil biochemistry, such as the high acidity, which is largely due to the woodland being classed as a pine forest. This may have caused a more hostile environment for certain pathogenic bacteria, meaning more prevalent soil organisms were able to out compete them and the cultivation of these pathogenic bacteria of interest was less likely.

Overall, these results show the ability to isolate APO from Friston Forest at the sample locations used, as well as the ability to locate organisms belonging to the *Streptomyces* genus. This includes those with the ability to produce inhibitory compounds, affecting both Gram-positive and Gram-negative test organisms. As well as this, the lack of pathogenic organisms, of those previously selected, from the several areas of Friston Forest sampled, was noted.

5.0 Investigation of soil samples from Dawes Farm

5.0 Investigation of soil samples from Dawes Farm

Similar to the New Forest, Dawes Farm was selected to allow for any effects that livestock have on the soil microbiome to be studied. In collaboration with Westpoint Farm Vets, who own the on-site practice, sampling again occurred at varying areas. This included grazing fields with animals, including cattle, present, and those where animals were previously present (relocated), as well as areas primarily affected by human activity, including walkways, to allow any differences to be noted. Westpoint Farm Vets specialise in the treatment and upkeep of farm animals, and hence differences may be seen compared to that of the New Forest results, in both presence of pathogenic organisms and APO, due to the differing animals and nature of the sites sampled; this may be due to the larger and more dense populations of animals affecting the soil microbiome, as well as the higher likelihood of antimicrobial use on Dawes Farm, due to the nature of the site. Several areas of Dawes Farm were sampled to increase the chances of isolating both potentially pathogenic organisms, as well as APO.

5.1 Methods

5.1.1 Isolation and characterisation of microorganisms within the soil

Soil samples were collected from several different locations at Dawes Farm, Warnham, in collaboration with Westpoint Farm Vets, and pathogenic organisms were isolated using the methods stated in section 2.2.1 and 2.2.2.2 respectively. For further characterisation, using biochemical tests, of the potential pathogenic bacteria, methods were carried out as in section 2.2.3.2.

For the isolation of potential APO and *Streptomyces* spp. methods were carried out as seen in section 2.2.2.3, with the use of both oat and potato starch agar as cultivation media. Further characterisation occurred using Gram staining and PCR as seen in section 2.2.3.3.

5.1.2 Further testing of Antimicrobial Producing Organisms

All APO that were cultivated using both potato starch and oat agar, were further tested using the perpendicular screening method, using both sensitive and clinical strains, as stated in sections 2.2.4.2 and 2.2.4.3 respectively, and then further tested using the starvation method, against sensitive strains of *E. coli*, *S. epidermidis*, *S. aureus* and *P. mirabilis* as seen in section 2.2.4.5. Repeats of perpendicular screening using sensitive strains took place only.

As well as this, Ms Suzana Haxhia (MSc Biomedical Science Project student) carried out the perpendicular method as seen in section 2.2.4.2, on presumed *Streptomyces* spp. that were isolated

from wet soil samples, originating from Dawes Farm only, for further determination of *Streptomyces* activity.

5.2 Results

Twenty-two soil samples were collected at Westpoint Farm Vets, Dawes Farm, Warnham, in May 2017 (See appendix table A8).

5.2.1 Isolation of pathogenic microorganisms

Ninety organisms were isolated and purified from the 22 soil samples taken from farmland belonging to Westpoint Farm Vets. Further testing including Gram staining, catalase, indole, coagulase, lactose fermentation, oxidase, and citrate, and the identification of isolates was carried out with assistance of project student Ms. Suzana Haxhia. From the farm samples 90 isolates were identified using biochemical tests; four isolates were identified as belonging to the *Klebsiella* spp., and *E. coli* (4.4%). Nineteen, 26, and six isolates were identified as *S. aureus* (21.1%), *Bacillus* spp. (28.9%), and *P. aeruginosa* (6.7%) respectively. One isolate was identified as *S. epidermidis* (1.1%), due to coagulase result. Eleven isolates were identified as belonging to the *Streptomyces* genus due to Gram stain (12.2%), and 19 isolates were identified as *Aureobacterium* spp. (21.1%).

5.2.2 Identification of *Streptomyces* spp. from APO isolated

From the 22 dried soil samples, 26 APO were isolated using oat agar, and 17 were isolated using potato starch agar (see appendix Tables A9 and A10). Using the Gram stain information of APO isolated using oat and potato starch agar, 11 (42.3%) and nine (52.9%) APO respectively were selected for further identification using DNA extraction and PCR; appendix Tables A11 and A12 display the concentration and the purity of the DNA extracted from all isolates. However, isolates were not put through conclusive PCR for the identification of *Streptomyces* spp., due to time constraints.

5.2.3 Testing of Antimicrobial Producing Organisms

5.2.3.1 Antimicrobial screening against sensitive organisms

All 26 and 17 APO isolated and purified from oat and potato starch agar respectively, due to apparent inhibitory effects and/or similar morphology to that of *Streptomyces* spp., were tested (see appendix Tables A9 and A10). From initial perpendicular screening ten out of the 26 oat isolates displayed inhibition to at least one of the test bacteria used (38.5%) (Table 5.1). Repeats of perpendicular screening using the oat isolates, originating from Dawes Farm, that had shown inhibition prior, displayed four out of the ten APO with continued inhibition (40%); isolates 112, 113, 115, 121, 123, and 126 displayed no inhibition upon the repeat of perpendicular screening

(Table 5.2). Repeated inhibition by APO, out of all oat isolates in total, was seen by 15.4% of isolates.

Three out of the 17 (17.6%) APO isolated from Dawes Farm, Warnham, using potato starch agar, were seen to present inhibition against at least one of the five test isolates in initial perpendicular screening (Table 5.3). All three isolates (100%) again showed inhibition on varying days, upon the repeat of this method, but inhibition was seen against less organisms than initially (Table 5.4); neither 131 or 132 presented inhibition against *P. mirabilis* with 132 also lacking inhibition against *S. aureus*. Isolate 142 also displayed inhibition against *S. epidermidis* only, upon repeats (Tables 5.3 and 5.4).

Of the 43 APO isolated using both agars, 13 (30.2%) displayed some form of inhibition to the sensitive strains of test bacteria. No inhibition was seen by either of the groups of isolates towards *P. aeruginosa*.

Table 5.1: The ten APO originally isolated using oat agar, from Dawes Farm, that displayed inhibition in initial perpendicular screening. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
105	-	*S. epidermidis*, *S. aureus*, and *P. mirabilis*.	-	-
112	-	*P. mirabilis*	-	*P. mirabilis*
113	-	-	-	*E. coli, S. epidermidis* and *S. aureus*.
115	-	*S. epidermidis* and *S. aureus*.	-	-
116	-	*S. epidermidis* and *S. aureus*.	-	-
120	-	*P. mirabilis*.	-	-
121	-	-	-	*P. mirabilis*
123	-	-	-	*S. epidermidis* and *S. aureus*.
126	-	*S. epidermidis* and *S. aureus*.	-	-
128	-	*S. epidermidis* and *S. aureus*.	-	-

Table 5.2: The inhibition seen using the repeat of the perpendicular screening method, by the ten APO originally isolated using oat agar, from Dawes Farm, Warnham, that had shown inhibition previously. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number:	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
105	-	*S. epidermidis*, *S. aureus* and *P. mirabilis*	-	-
112	-	-	-	-
113	-	-	-	-
115	-	-	-	-
116	-	*S. epidermidis* and *S. aureus.*	-	-
120	-	-	-	*P. mirabilis*
121	-	-	-	-
123	-	-	-	-
126	-	-	-	-
128	-	*S. aureus*	-	-

Table 5.3: The inhibition seen by the three APO isolated originally from Dawes Farm using potato starch agar, when tested using the perpendicular screening method. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number	Inhibition of test bacteria seen at 25°C		Inhibition if test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
131	-	*S. epidermidis, S. aureus* and *P. mirabilis*	-	-
132	-	*S. epidermidis*, *S. aureus* and *P. mirabilis*	-	-
142	-	-	-	*E. coli*, *S. epidermidis* and *S. aureus*.

Table 5.4: The inhibition seen by the three APO with inhibitory activity seen previously, isolated from Dawes Farm, Warnham, using potato starch agar, upon the repeat of the perpendicular screening method. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
	0 day	2 day	0 day	2 day
131	-	*S. epidermidis* and *S. aureus*	-	-
132	-	*S. epidermidis*	-	-
142	-	-	-	*S. epidermidis*

5.2.3.2 Antimicrobial screening against clinical strains

Out of 10 APO originally isolated from Dawes Farm, and cultivated using oat agar, that showed inhibition prior (Table 5.1), three were seen to have inhibitory activity towards at least one of the clinical test bacteria using the perpendicular screening method (30%) (Table 5.5). Isolate 105 showed inhibition against *S. aureus* and *P. mirabilis*, with inhibition towards *P. mirabilis* shown in figure 5.1. Two of the three (66.6%) potato starch isolates from Dawes Farm, that had shown inhibition prior in initial screening, showed activity towards the clinically isolated bacteria used (Table 5.6). Figure 5.2 displays the inhibition towards *S. aureus* and *P. mirabilis* by isolate 132, however due to the overgrowth of *Proteus* spp. on this plate, confirmation of inhibitory action against MRSA was not confirmed using this replicate.

None of the APO isolated using the two agars were seen to be effective against clinically isolated *E. coli*; two of the isolates, 113 and 142, had shown inhibition of sensitive *E. coli* previously (Tables 5.1 and 5.3). Totally, inhibition towards clinical strains of test bacteria, was seen by five (38.5%) out of the 13 APO, that had previously shown inhibition.

Table 5.5: Inhibition shown towards clinically isolated test bacteria, using perpendicular screening, by the ten APO initially isolated from Dawes Farm using oat agar, that showed inhibition previously. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	Test Organisms used	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
		0 day	2 day	0 day	2 day
105	*S. aureus* and *P. mirabilis*	-	*S. aureus* and *P. mirabilis.*	-	-
112	*P. mirabilis*	-	-	-	-
113	*E. coli* and *S. aureus.*		-		-
115	*S. aureus*	-	*S. aureus*.	-	-
116	*S. aureus*	-	-	-	-
120	*P. mirabilis*	-	-	-	*P. mirabilis*
121	*P. mirabilis*	-	-	-	-
123	*S. aureus*	-	-	-	-
126	*S. aureus*	-	-	-	-
128	*S. aureus*	-	-	-	-

Table 5.6: Inhibition seen by the three APO, that were initially cultivated using potato starch agar from Dawes Farm, towards clinically isolated test bacteria in perpendicular screening. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate number	Test Organisms used	Inhibition of test bacteria seen at 25°C		Inhibition of test bacteria seen at 37°C	
		0 day	2 day	0 day	2 day
131	*S. aureus* and *P. mirabilis*	-	*P. mirabilis* and *S. aureus*.	-	-
132	*S. aureus* and *P. mirabilis*	-	*P. mirabilis* and *S. aureus*	-	-
142	*E. coli* and *S. aureus.*	-	-	-	-

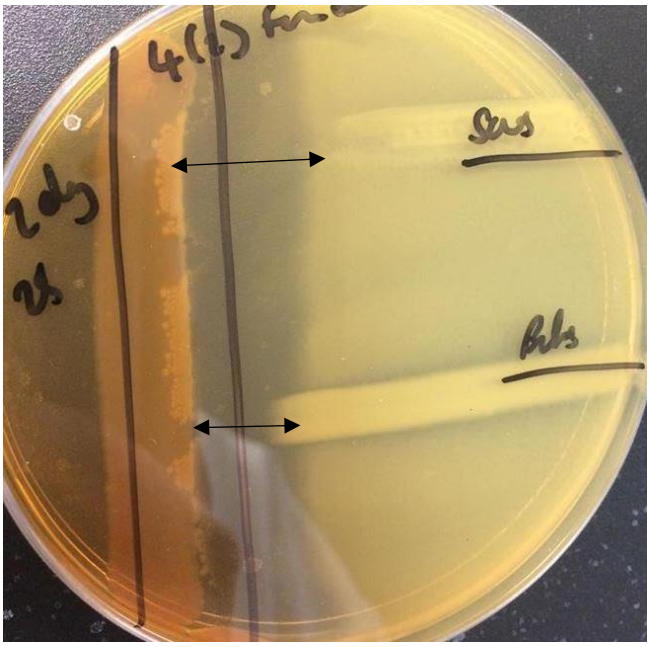

Figure 5.1: Isolate 105, isolated from Dawes Farm using oat agar, displaying inhibition against clinically isolated *S. aureus* (top) and *P. mirabilis* (bottom). Isolate 105 was incubated 2 days prior the addition of test isolates at 25°C. The arrows display the inhibitory activity of 105 towards the test organisms used. However, from this figure, activity towards MRSA could not be confirmed, due to the overgrowth of *P. mirabilis*.

Figure 5.2: The inhibitory activity of isolate 132 isolated using potato starch agar from Dawes Farm, against clinically isolated *S. aureus* (top) and *P. mirabilis* (bottom) after previous incubation of this organism for 2 days at 25°C, using the perpendicular screening method. The arrows indicate where the inhibition of the test organisms has taken place. However due to the overgrowth of the *Proteus* spp., the inhibition of *S. aureus* was not confirmed using this plate replicate.

5.2.3.3 Starvation method

All 26 and 17 isolates cultivated originally from oat and potato agar respectively, were further tested using prolonged incubation at 25°C and 37°C, using the starvation method. Out of the 26 oat isolates, five displayed inhibition to at least one of the test bacteria used (19.2%) (Table 5.7). Figure 5.3 displays the inhibition seen towards *E. coli* by oat isolate 112 after incubation for 10 days at 37°C. Figure 5.4 displays inhibitory action towards *S. aureus* after 7-day incubation of oat isolate 120 at 37°C.

Four out of the 17 potato isolates were seen to present inhibitory activity towards the test bacteria used, after prolonged incubation during the starvation method (23.5%) (Table 5.8). Figures 5.5 and 5.6 display inhibition against *S. aureus* by isolates 146 and 136 respectively, after both isolates were incubated for 10 days at 37°C. In total nine out of the 43 (20.9%) farm isolates, cultivated using both agars, displayed inhibition towards one or more of the test bacteria.

Within the oat isolates, inhibition was seen against all test bacteria used, including *P. mirabilis*, however isolates initially cultivated using potato starch agar, presented no inhibition towards the Gram-negative test bacteria used; no inhibitory activity was seen towards *E. coli* or *P. mirabilis* within the varying temperatures and incubation periods (Tables 5.7 and 5.8).

Table 5.7: The inhibition seen in the starvation method by the APO isolated from Dawes Farm, using oat agar as the cultivation media. Agar results in brackets – PC – plate count agar, MH – Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number	Days of incubation of isolate at 25°C (agar result in brackets)			Days of incubation of isolate at 37°C (agar result in brackets).		
	7 days	10 days	14 days	7 days	10 days	14 days
112	-	-	-	*E. coli* (MH), *P. mirabilis* (PC).	*E. coli*, *S. aureus* and *S. epidermidis* (PC), *P. mirabilis* (MH).	*S. aureus* and *S. epidermidis* (PC) and *E. coli* (MH).
114	*S. aureus* and *S. epidermidis*.	-	-	-	-	-
120	-	-	-	*E. coli, S. epidermidis* and *S. aureus* (MH).	-	*S. epidermidis* and *S. aureus* (PC), *E. coli* and *P. mirabilis* (MH).
121	-	-	-	-	*E. coli* (MH).	*S. epidermidis* (PC).
129	-	-	-	-	*E. coli*, *S. epidermidis* and *S. aureus* (MH).	-

Table 5.8: Inhibition seen by the APO isolated from Dawes Farm, using potato starch agar as initial cultivation media, using the starvation method. Agar results in brackets – PC – plate count agar, MH – Mueller-Hinton agar. A (-) indicates no inhibition of test organisms was apparent under the conditions stated.

Isolate Number	Days incubation of isolate at 25°C (Agar result in brackets)			Days incubation of isolate at 37°C (Agar result in brackets)		
	7 days	10 days	14 days	7 days	10 days	14 days
136	-	-	-	-	*S. aureus* and *S. epidermidis* (PC).	-
137	-	*S. epidermidis* (PC)	*S. epidermidis* and *S. aureus* (PC).	-	-	-
144	-	*S. epidermidis* and *S. aureus* (MH).	-	-	-	-
146	-	-	-	-	*S. epidermidis* and *S. aureus* (PC).	-

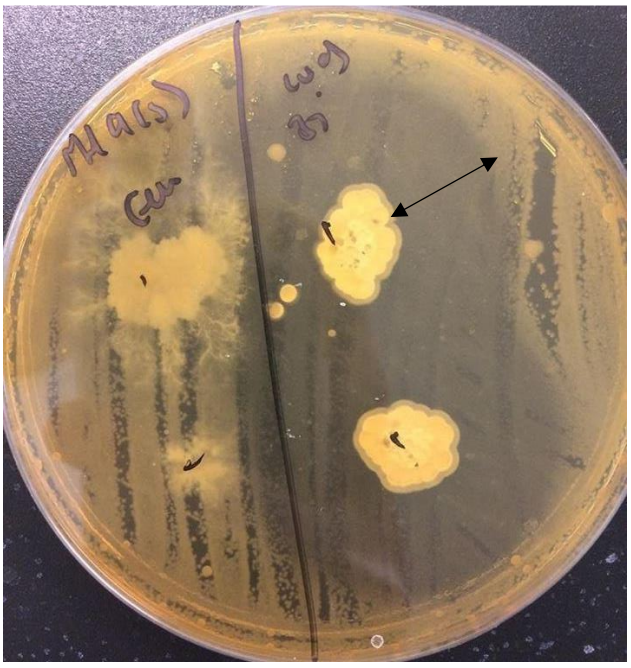

Figure 5.3: Isolate 112 isolated from Dawes Farm, using oat agar, displaying inhibition against *E. coli,* after 10-day incubation at 37°C, using the starvation method. The arrow displays an example of the extent of inhibition of *E. coli* by APO 112.

Figure 5.4: Isolate 120 isolated from Dawes Farm using oat, displaying inhibition against *S. aureus*, on Mueller-Hinton agar, after prolonged incubation at 37°C for 7 days, using the starvation method. The arrow displays where the growth of *S. aureus* has been inhibited.

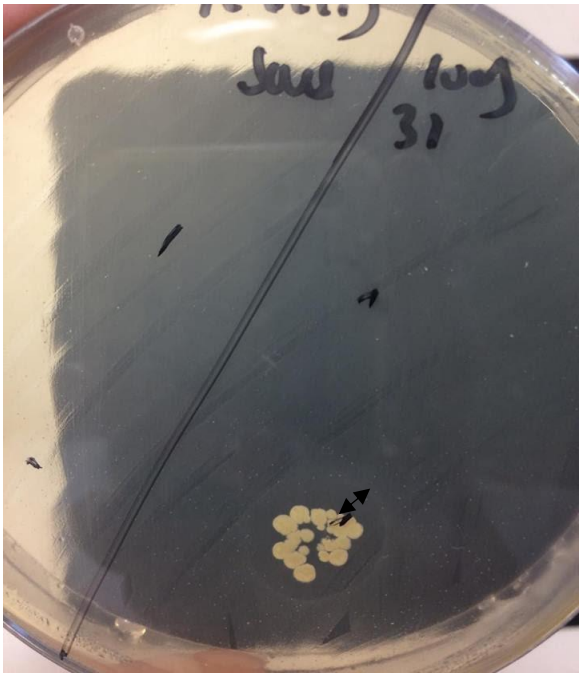

Figure 5.5: Inhibition against *S. aureus* on plate count agar, by Dawes Farm potato isolate 146, after incubation for 10 days at 37°C, using the starvation method. The arrow displays the zone of inhibition seen against *S. aureus*.

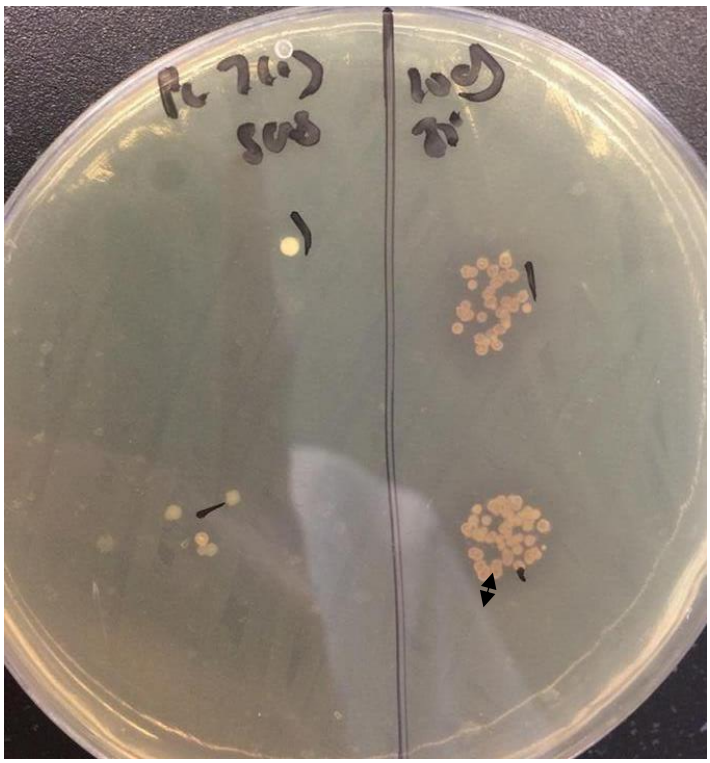

Figure 5.6: Dawes Farm isolate 136 that was initially cultivated using potato starch agar displaying inhibition against *S. aureus* on plate count agar, after incubation at 37°C for 10 days in the starvation method. The black arrow displays an area of inhibition of *S. aureus*.

5.3 Discussion

Dawes Farm was one of the sample locations selected for the isolation of pathogenic bacteria, and organisms with the ability to produce inhibitory compounds, effective against other bacterial organisms. In collaboration with Dr Tim Potter, and Westpoint Farm Vets, 22 samples of soil were taken in and around grazing fields, where animals were or had been present, resulting in the isolation of 90 organisms, with further identification carried out by Ms. Suzana Haxhia. From this work, several of the pathogenic organisms, that had been previously selected in section 2.2.2.2, were isolated including four (4.4%) *E. coli* and *Klebsiella* spp. The majority of organisms, however, were identified as belonging to the *Bacillus* spp., with 26 (28.9%) identified as belonging to this genus, followed by 19 (21.1%) identified as *S. aureus*. As well as this one organism (1.1%) was said to be *S. epidermidis* and 11 (12.2%) *Streptomyces* spp. were isolated. Although these are not included in the specific organisms we aimed to locate in section 2.2.2.2, such organisms as *S. aureus* may provide additional information of the sample areas and can still be determined as pathogenic, due to their known ability to cause skin and wound infections, with this organism also selected as one of the organisms presenting international concern due to its resistance (WHO, 2014). As well as the isolation of these organisms, it was also noted that six (6.7%) *P. aeruginosa* were isolated, which provides further evidence of the variety of pathogenic organisms that may be present in the soil, due to its known resistance as one of the ESKAPE pathogens (Rice, 2008; Santajit and Indrawattana, 2016).

The location of these pathogenic organisms, including *E. coli* and *P. aeruginosa* may be explained by the location properties. All samples were collected at the farmland, which included areas where cattle resided or were currently present, which may be thought to have an effect on the overall soil microbiome, hence increasing the likelihood of certain pathogenic organisms being isolated (Table A8). However, the collection of more samples from the same and differing areas within Dawes Farm would allow confirmation of this conclusion, as well as allowing further assumptions regarding the presence and activities of APO from the same locations to be made.

As well as the identification of isolates, Ms Haxhia also carried out perpendicular screening (as seen in section 2.2.4.2) on the isolates that were identified as belonging to the *Streptomyces* spp., from the wet soil samples, to increase the likelihood for the identification of novel inhibitory compounds. However, none of these isolates were seen to present inhibitory activity to the sensitive test organisms used, and it hence may be determined that the ability of *Streptomyces,* for the production of novel antimicrobials, has been exhausted.

As well as the *Streptomyces* isolated from the wet soil samples discussed previously, PCR was intended to be undertaken for the identification of this genus from the APO collected, allowing further conclusions to be drawn regarding these isolates inhibitory activity. However, due to time

constraints, this method was not carried out, meaning isolates can only be presumed as belonging to this genus by morphology, and cell characteristics, including Gram stain. Out of the 43 APO isolated from soil samples using both oat and potato starch agar, 20 (46.5%) of these were presumed as belonging to the *Streptomyces* spp.; 11 (42.3%) isolated using oat agar and nine (52.9%) isolated using potato starch agar (see appendix tables A11 and A12). From the presumed *Streptomyces* isolated using oat agar, isolates 115, 121, 123, 126 and 128 all displayed inhibition in perpendicular screening, with isolate 121 displaying continued inhibition in the starvation method, and isolate 115 displaying inhibitory activity against MRSA (Tables 5.1, 5.2, 5.5, and 5.7). However, all the presumed *Streptomyces* from the potato agar isolates, displayed inhibition only in the starvation method, with isolates 137, 144 and 146 displaying inhibitory activity (Tables 5.3 and 5.8). Future work to confirm these isolates identification however, would allow the further determination of antimicrobial activity of the *Streptomyces* isolates cultivated from Dawes Farm, of both those collected as APO, and the 11 isolates collected from the wet soil samples.

As well as inhibition that was seen by the presumed *Streptomyces* spp., as discussed above, further inhibition was seen by additional isolates in the differing methods. In total, 26 and 17 APO were isolated from oat and potato starch agar, with 10 (38.5%) and three (17.6%) displaying inhibition respectively, in perpendicular screening (Tables 5.1 and 5.3). Within this screening several isolates were also seen to present inhibition towards the sensitive strain of *P. mirabilis*; three oat isolates (11.5%), and two (11.8%) APO isolated using potato starch agar, presented inhibition of this organism. A large amount of APO were also seen to present inhibition to the Gram-positive test organisms used, with 10 of the 13 (76.9%) confirmed APO from Dawes Farm presenting inhibition to one or both of the *Staphylococcus* spp. However, no inhibition was seen towards the strain of *P. aeruginosa* used, by any of the APO, potentially displaying the lack of antimicrobial compounds within the soil with effect on this organism.

Upon further testing of these isolates using the clinical strains of the test bacteria, further inhibition was seen by three (30%) and two (66.6%) APO originally cultivated using oat and potato starch agar respectively, with a total of five (38.5%) displaying inhibitory activity towards one of the clinical strains (Tables 5.5 and 5.6). Although it was shown that none of the antimicrobial compounds being produced were able to inhibit the growth of the clinical *E. coli* strain, inhibition was seen towards the clinical strain of *P. mirabilis* and MRSA, of which three isolates, 105, 131 and 132 were seen to present inhibition towards both of these organisms (Tables 5.5 and 5.6). Figure 5.1 displays the activity of isolate 105 against *P. mirabilis*, with inhibition towards MRSA also caused by this APO, which may highlight this organism as especially useful. Future work may hence focus on the determination of this organism's genus, as well as the active compound being produced, in the hope of locating a new antimicrobial, able to contribute to overcoming the resistance currently being presented by several organisms, and the existing AMR crisis (WHO, 2014).

Additional to the perpendicular screening carried out, the starvation method, using *S. aureus*, *S. epidermidis*, *E. coli* and *P. mirabilis* as test organisms, was used to increase antimicrobial activity of the APO isolated. However, this method was not seen to greatly increase the amounts of inhibition seen by isolates. Of the 17 APO isolated using potato starch agar, four (23.5%) of the isolates were seen to be inhibitory compared to three (17.6%) seen in initial screening (Tables 5.3 and 5.8), with less inhibition seen by oat isolates in the starvation method; only five of the 26 isolates (19.2%) displayed inhibitory activity in the starvation method (Table 5.7), compared to 10 isolates (38.5%) with inhibition in initial perpendicular screening (Table 5.1), showing a decrease of inhibition by 50%. This displays an overall decrease of inhibition from initial screening of around 10%, with 30.2% of organisms displaying inhibition in perpendicular screening compared to only 20.9% in the starvation method. An explanation for this may be due to the stress-induced conditions inhibiting the growth of certain APO and/or preventing them from producing their antimicrobial compounds of interest, due to the lack of nutrients available. However, only four test organisms were used for the testing of inhibitory activity by these APO in the starvation method, and hence for future work the inclusion of more test organisms, both Gram-positive and Gram-negative bacteria, as well as the potential for pathogenic fungal strains to be included, would provide more conclusions about the antimicrobial activity of the compounds being produced by the differing APO. On the other hand, the same sensitive test organisms were used in initial perpendicular screening, so the amount of test organisms used, should not have affected the decrease in inhibition seen between the methods.

Although a decrease of overall inhibition was seen, isolates with no prior inhibition were seen to be active in the starvation method; APO 114 and 129, detailed inhibition in the starvation method against *S. aureus* and *S. epidermidis*, and *E. coli* and both *Staphylococcus* spp. after incubation for 7 days at 25°C and 10 days at 37°C respectively (Tables 5.1 and 5.7). This may demonstrate the starvation method as being an effective way to stress the organisms into producing novel antimicrobial compounds of interest. These results also again display the large amounts of inhibitory activity seen towards the Gram-positive test organisms, as discussed with the results seen in initial screening, with all APO presenting inhibition in the starvation method, producing inhibitory activity to at least one of the *Staphylococcus* spp. (Tables 5.7 and 5.8).

From this further testing of all APO using the methods, some APO can be seen as extremely versatile in their ability to produce inhibitory compounds. Isolates 112, 120 and 121, displayed inhibitory activity in both perpendicular screening and the starvation method, with APO 112 seen to produce inhibition against *P. mirabilis* at both temperatures, after 2-day incubation in initial screening, and 120 displaying the ability to inhibit the clinical strain of *P. mirabilis* (Tables 5.1, 5.5 and 5.7). It may hence be favourable to take forward these isolates for further testing and characterisation, as well as determining whether the active compounds being produced, are novel

and of interest. However, apart from isolate 112, all other isolates lacked the ability to inhibit test organisms at both test temperatures used. On the other hand, isolate 112 and others including isolates 120, 121 and 137 were able to produce inhibitory compounds after at least two of the differing day incubations in the starvation method, at either 25°C or 37°C (Tables 5.7 and 5.8).

Overall, Dawes Farm, Warnham, can be confirmed as a location from which pathogenic organisms, and APO can be isolated from, including those with activity against both the *Staphylococcus* spp. and *Proteus* spp., as well as the clinical strains of *P. mirabilis* and MRSA, displaying the potential for the isolation of a novel antimicrobial, able to contribute to the fight against AMR, from this location.

6.0 Discussion

6.0 Discussion

AMR has been seen to develop all around the world, with several of the last resort antibiotics being determined as ineffective (O'Neill, 2016). This has and will continue to compromise our healthcare systems as we know it, with it necessary to tackle antibiotic resistance using several different approaches (WHO, 2015). Increasing awareness of antibiotic misuse and resistance, as well as the research and development of novel antimicrobials to treat organisms presenting resistance, are just two examples of those proposed to combat this issue (WHO, 2016; O'Neill 2016). This study focusses on the latter, by forwarding the research needed into the isolation and characterisation of Antimicrobial Producing Organisms (APO) from previously untested environments.

The location and identification of Actinomycetes, and the presence of novel inhibitory compounds in samples of soil, has been the subject of several studies around the world. Some studies have focussed on soils from hot geographical locations. Singh and colleagues (2012), isolated seven Actinomycetes from soil samples from several areas in Gwalior, India, with one isolate displaying antimicrobial activity against both Gram-negative and Gram-positive bacteria, including activity against a vancomycin resistant enterococci test strain. However, the present study focusses on a more temperate environment, and as far as it is known is the first study focusing on the location of antimicrobial compounds from both Friston Forest and New Forest with the help of the Forestry Commission UK, and specific areas of Dawes Farmland at Warnham, West Sussex, in collaboration with Westpoint Farm Vet group.

Soil organisms, including the *Streptomyces* spp., show great potential in their ability to produce novel secondary metabolites, with almost two thirds of antibiotics that are naturally occurring, being produced by this genus (Mohanraj and Sekar, 2013). In this study, the cultivation of *Streptomyces* spp., as well as other soil organisms presenting inhibitory ability, was carried out with the view of isolating novel antimicrobial compounds. A total of 84 soil samples were taken from the New Forest (42), Friston Forest (20), and Dawes Farm (22), greater than the number of samples collected by Ganesan and colleagues (2016), who collected soil samples from five different places in Tamil Nadu, India. This provided a range of samples and sample locations in which novel compounds, and the parent organisms may be isolated. In total 146 APO were isolated for screening with 34, 69, and 43 APO isolated from the New Forest, Friston Forest and Dawes Farm, respectively, due to displayed inhibitory activity, and/or morphology similar to that of *Streptomyces* spp.

Using these APO, it was confirmed through testing that at least one isolate originating from each of the differing sample locations showed the ability to inhibit other test organisms of importance. From initial screening, five (14.7%) out of the 34 New Forest isolates displayed inhibition against *S. epidermidis*, *S. aureus*, and/or *E. coli,* with no inhibition seen towards *P.*

aeruginosa or *P. mirabilis* (Table 3.2). No inhibition was again seen towards *P. aeruginosa* by either Friston Forest or Dawes Farm APO, however inhibitory activity towards *P. mirabilis* was presented by APO isolated from these locations. This may be due to the known low antibiotic susceptibility of *P. aeruginosa*, as well as the organism's ability to rapidly develop intrinsic resistance towards antibiotics (Hancock and Speert, 2000; Lister *et al.,* 2009). Overall 15.9% and 30.2% of APO isolated from Friston Forest and Dawes Farm inhibited sensitive strains of test bacteria in initial perpendicular screening respectively, with Friston isolate 60 and five of the APO isolated from Dawes Farm inhibiting the growth of the sensitive strain of *P. mirabilis* a well-known opportunistic pathogen in humans and a common cause of urinary tract infections (Tables 4.2, 4.3, 4.4, 5.1 and 5.3) (Chen *et al.,* 2012).

Much of the inhibition seen using the perpendicular screening method however, was seen against the Gram-positive organisms *S. epidermidis* and/or *S. aureus*. Four isolates out of the five (80%) confirmed APO from New Forest (Table 3.2) and 10 out of the 11 (90.9%) Friston Forest APO, displayed inhibition towards at least one of the Gram-positive test bacteria used as discussed in section 3.3 and 4.3. This was a recurring theme also seen from Dawes Farm APO strains discussed in section 5.3, with 10 out of the 13 (76.9%) inhibitory APO, showing inhibition to at least one of the Gram-positive organisms used.

Perpendicular screening, has been used by several other studies as a viable method for the determination of inhibitory activity of isolated organisms, including those belonging to the Actinomycetes order (Bizuye *et al.,* 2013; Singh *et al.,* 2016). Using this method, Gebreyohannes and colleagues (2013) found that 13 out of 31 collected Actinomycetes strains displayed inhibitory activity to at least one of the test bacteria used, including inhibition seen towards both *P. aeruginosa* and *S.* Typhimurium. However, their study investigated the antimicrobial activity of Actinomycete isolates only. This may highlight a strongpoint of the present study, in which all organisms with observable zones of inhibition in initial cultivation, plus those with characteristics similar to that of the *Streptomyces* genus, were selected for further testing, increasing the likelihood of novel antimicrobial compounds being discovered.

Further testing of inhibitory activity of APO isolated from both Friston Forest and Dawes Farm, was carried out using the perpendicular screening method, with clinical strains of *E. coli*, MRSA and *P. mirabilis* used to determine the activity of the APO isolated further. Antibiograms were carried out on these clinical strains to identify any resistance they possessed (Table A13). *P. mirabilis* displayed sensitivity to all antibiotics used, however both *E. coli* and MRSA showed some form of resistance to at least one of the antibiotics. *E. coli* displayed resistance to both amoxicillin and clavulanic acid (30µg), and gentamycin (10µg), as well as intermediate sensitively to cefotaxime (5µg). MRSA displayed resistance towards erythromycin (5µg) only out of the antibiotics used, with

it assumed to confer resistance to the β-lactams class of antibiotics, due to its classification as a methicillin-resistant organism (Table A13).

Similar amounts of inhibition was seen by APO towards the clinical strains of test bacteria isolated from Friston and Dawes Farm, with 33% and 38.5% of isolates displaying inhibition towards at least one of the strains of test bacteria (Tables 4.6 and 5.5). However, compared to the inhibition seen by the Friston Forest isolates, the Dawes Farm APO detailed no activity against *E. coli*. Conversely four isolates did show inhibition towards MRSA, and a greater amount of inhibition towards the clinical strain of *P. mirabilis* was seen, although it should be noted that Dawes Farm APO had greater inhibition towards the sensitive *Proteus* test strain in initial screening compared to Friston APO, as discussed in sections 4.3 and 5.3. These results provide further evidence for the presence of APO in the soil, as well as confirming the ability to isolate APO from these two locations with inhibitory activity against clinical strains of bacteria. A clinical strain of *S. epidermidis* was not available for use in the further testing using the perpendicular screening method, and hence future work could include the repeat of this method using this organism.

Similar to the results seen previously in perpendicular screening, and discussed above and in sections, 3.3, 4.3 and 5.3, large amounts of inhibition were again seen against Gram-positive bacteria in the starvation method, with 91.7% and 61.9% of confirmed APO from New Forest and Friston Forest respectively, displaying inhibition towards at least one of the Gram-positive test strains used. As well as this, all Dawes Farm APO confirmed to have inhibitory activity in the starvation method, displayed inhibition towards at least one of the Gram-positive test organisms used, as discussed in section 5.3. It has been hypothesized that this may be due to membrane structure, in which Gram-positive organisms lack an outer cell membrane containing lipopolysaccharide (LPS), which is known to make Gram-negative cell walls more hydrophobic and impermeable, meaning they are more susceptible to the inhibitory metabolites (Singh *et al.,* 2016; Kim *et al.,* 1994).

The more prominent inhibitory activity seen towards the Gram-positive bacteria in both the starvation and perpendicular screening methods, may highlight a lack of inhibitory compounds, able to affect the growth of Gram-negative bacteria, available for isolation from the soil. This was also seen by Ling and colleagues (2015), who using a device called the iChip, cultivated soil organisms, unculturable in lab conditions, within the soil itself, which resulted in the isolation of the new antibiotic, teixobactin. This antibiotic displayed good activity against a range of Gram-positive organisms, such as MRSA and *Streptococcus* spp. however, little inhibitory activity was seen towards Gram-negative organisms, such as *P. aeruginosa* and *Klebsiella pneumoniae* (Ling *et al.,* 2015). This highlights further, the research needed for the production of novel antibiotics, with the ability to inhibit the growth of Gram-negative organisms, especially those that are currently

presenting problematic high levels of resistance worldwide (Gulland, 2017, Nordmann *et al.,* 2009; Chen *et al.,* 2012).

The production of secondary metabolites, from primary metabolites, key for bacterial growth and reproduction in *Streptomyces*, as well as other organisms, is known to be highly complex (Rokem *et al.,* 2007). Several gene products, including those with enzymatic and regulatory functions, are involved in the control and production of these potentially antimicrobial metabolites (see figure 3.8) (Rokem *et al.,* 2007; Bekker *et al.,* 2015). It has also been shown that the expression of genes, involved in control of secondary metabolite production in Actinomycetes, is highly dependent on cultivation conditions, and often requires specific environmental changes and/or starvation, for certain metabolites to be produced (Liu *et al.,* 2013; Gullo *et al.,* 2006). The starvation method presented in this study was employed with the aim of increasing the amount of inhibition produced by the APO, compared to initial screening, by varying the cultivation conditions, increasing the likelihood of a bacterial stress response, and hence production of novel antimicrobials (Gullo *et al.,* 2006).

The starvation method was shown to be successful in increasing the amounts of inhibitory activity produced by APO with both New Forest and Friston Forest isolates as previously discussed in sections 3.3. and 4.3. However, overall nearly a 10% decrease was seen in the total amount of Dawes Farm APO, presenting inhibition in the starvation method, compared to that seen in perpendicular screening as discussed in section 5.3. This decrease of isolates displaying inhibition may due to the stress-inducing conditions used, preventing the growth of the APO during incubation, or causing cell death due to a lack of nutrients. On the other hand, even with this decrease in the number of isolates displaying inhibition, several organisms with no previous inhibitory activity displayed in the novel antimicrobial screening method, presented inhibition towards test organisms in the starvation method, highlighting the ability of this method at inducing the production of inhibitory compounds from certain APO (see sections 4.3 and 5.3).

Using this starvation method, certain isolates also displayed the ability to produce inhibitory compounds under a variety of conditions, including production after several days incubation, as well as at both of the differing temperatures used. Friston Forest isolates 53, 70, 73 and 78, as well as New Forest APO 26 were all seen to produce inhibitory compounds towards test organisms, under a range of different conditions, used in this method (see Sections 3.3 and 4.3). However, none of the APO isolated from Dawes Farm were seen to present inhibition at both temperatures used, (25°C and 37°C), in the starvation method (Tables 5.7 and 5.8), displaying a large difference in the amount of inhibition presented by farm APO, compared to that seen by APO cultivated from the forest samples. This may highlight forest land as a more prolific producer of

APO, and hence more favourable in the search of novel antimicrobials. Contrastingly, APO 112 isolated from Dawes Farm, was seen to show inhibition towards *P. mirabilis* after 2 days incubation, at both temperatures, in initial perpendicular screening (Table 5.1), however this may be determined as not comparable to the forest inhibition results, as both locations also detailed APO with the ability to inhibit test organisms, at both of the temperatures used in this method, as discussed in sections 3.3 and 4.3.

Correlation of the inhibition seen spanning all methods, details the capabilities of certain APO that showed repeated inhibition, for producing inhibitory compounds under a variety of conditions. This was seen by APO 16, 26, 53, 60, 70, 112, 120 and 121, isolated from all three sample locations, with both 60 and 120 also producing compounds able to inhibit the clinical strain of *P. mirabilis* (see sections 3.3, 4.3 and 5.3). The repeat of the starvation method, as well as the reuse of supernatants however, was not repeated for the Friston Forest and Dawes Farm isolates due to time constraints. Hence future work would allow for this to be carried out allowing further conclusions to be drawn, as well as comparison with the New Forest repeat results (Tables 3.3, 3.4 and 3.5). Improvements of methodology of the starvation method, as discussed in section 3.3, could also be carried out, including correct storage, and direct analysis using DOSY-NMR, of any supernatants with displayed inhibitory activity, as well as the addition of further organisms to the method, which may include clinical strains, and fungal isolates. To also allow further assumptions regarding APO activity, these additional test organisms may also be added to the perpendicular screening method, as seen by several other studies (Arifuzzaman *et al.,* 2010; Ganesan *et al.,* 2016). Ganesan and colleagues (2016), used 24 differing organisms in their initial perpendicular screening, which included both Gram-positive and Gram-negative bacteria, as well as pathogenic fungal strains, such as those belonging to the *Aspergillus* spp., detailing a much larger amount of test organisms used, compared to the present study.

For other future work, it too would then be favourable to take forward these APO, with shown prolific inhibitory ability, for further testing and characterisation. This could involve using more accurate molecular methods, such as DNA isolation and sequencing, which has been used by several other studies, to give a more representative view of the organisms, and their products of interest (Ganesan *et al.,* 2016; Ling *et al.,* 2015). This was seen by Kumar and colleagues (2014), who used DNA sequencing and phylogenetic analysis to identify the isolated organism presenting the most activity, which was confirmed as belonging to the *Streptomyces* spp. Atta and Ahmad (2009), also used DNA isolation and PCR amplification of the 16S rDNA gene, for phylogenetic analysis of an Actinomycete isolate which displayed potent anti-fungal activity. Sequencing of this 16S rDNA gene fragment allowed the organisms identity to be determined, which was confirmed as belonging to the *Streptomyces* genus (Atta and Ahmad, 2009). Hence, for future work the further purification and DNA sequencing of the 16S rRNA fragments, previously amplified in this study, may

provide further insights into the APO identity as well as any antibacterial compounds being produced.

From the dried soil samples, the APO were selected due to displayed inhibition, and/or similar morphology to that of *Streptomyces* spp., with this genus being actively selected for due to the initial cultivation media used in the present study. This was also seen in the study by Kumar and colleagues (2014), who actively selected for this genus, and focussed on these organisms for the presence of any inhibitory activity against test organisms. However, due to the selection of all isolates that displayed any form of any inhibition, this current study worked to identify those belonging to the *Streptomyces* spp. within these APO isolated, using Gram stain as presumptive identification, and PCR for confirmation of isolates belonging to this species. Several isolates were presumed as belonging to this genus by its long-branched Gram-positive rods, seen using Gram stain carried out prior to PCR as discussed in sections 4.3 and 5.3. In total 19 isolates were confirmed as belonging to the *Streptomyces* spp., all identified from Friston Forest samples, with eight (42.1%) of them displaying some form of inhibitory activity. However, due to time constraints, this process was halted, leaving the identification of some of the *Streptomyces* spp. as presumptive.

As discussed previously correlation can be drawn between the isolates confirmed as belonging to the *Streptomyces* spp., and the methods used to encourage the inhibitory activity of the APO. For example, isolate 49, that was confirmed as belonging to the *Streptomyces* spp., was seen to produce inhibition in both repeats of the perpendicular screening method, however as discussed in section 4.3, isolate 49 displayed no further inhibition towards any of the clinical strains of test bacteria used. This may highlight the lack of inhibitory activity, of compounds produced from the *Streptomyces* spp., against clinical and resistant isolates, possibly as a result of pathological fitness. Another hypothesis is that the inhibitory compound produced, is one of the vast amounts of known antimicrobial compounds, known for being produced by the *Streptomyces* genus, and hence has already been used for the treatment of infections, allowing organisms to have developed resistance against it (Miao and Davies, 2010). This too, may highlight that the isolation of novel inhibitory metabolites within the soil, has already been exhausted, and a differing approach may need to be developed (Rokem *et al.,* 2007). Confirmed *Streptomyces* isolates 76, 84, 96, 97, 98, 100 and 102 also displayed a lack of further inhibitory ability against the clinical strains, as discussed in section 4.3, providing further evidence for this hypothesis. It should be noted that isolates presumed to belong to the *Streptomyces* genus were seen to have activity towards the clinical strains of test bacteria, as discussed in sections 4.3 and 5.3. However these isolates, as well as other APO isolated from both Friston, and New Forest, could only be presumed as belonging to the *Streptomyces* genus, hence continuation of the identification of these isolates using PCR would be beneficial in further work, to allow confirmation of whether the inhibitory compounds produced,

were a result of *Streptomyces* spp. known ability to produce vast amounts of biologically active secondary metabolites (Selvameenal *et al.,* 2009).

However, this study also noted that inhibition was additionally seen by isolates, not identified as belonging to the *Streptomyces* spp., including all of the New Forest APO, and several of the Dawes Farm, and Friston Forest isolates (see sections 3.3, 4.3 and 5.3). This provides positive evidence for the collection of all isolates presenting inhibition, not just those with similar morphology to bacteria belonging to the *Streptomyces* genus. A study by Mashoria and colleagues (2014), also selected for all organisms that displayed inhibition, for further testing, from the soil samples collected, similar to the present study.

This study has revealed the presence of APO from Friston Forest, the New Forest, as well as Farmland (Dawes Farm). Further study can determine if certain locations are more likely to present either APO and/or organisms belonging to the *Streptomyces* spp. This may help push forward the identification of organisms with the ability to produce inhibitory compounds, by highlighting specific locations and/or environmental factors, that may increase the probability of isolating APO. Using the differing location information, minor conclusions can be drawn. In initial perpendicular screening, three of the New Forest isolates from location 16, (isolates 9, 10 and 11) presented inhibition, and were initially isolated near cow faeces (Tables 3.2 and A1). Friston Forest isolate 64, displayed ability to inhibit clinical strains of test bacteria (Tables 4.2 and 4.6), was also cultivated from soil (sample 20) near rabbit faeces (see appendix table A3). As well as both of the forest results, it may be noted that any inhibition seen from APO collected from Dawes Farm, may have been due to the large amounts of animal activity, including isolate 129, initially isolated from a pile of faecal matter, which presented inhibition in the starvation method (Table 5.7). These results could indicate the positive effect that animals, and their faecal matter, have on the soil microbiome, increasing the likelihood for the isolation of APO, however a repeat collection and cultivation of the same, as well as differing areas with the same environmental characteristics, would allow further conclusions to be drawn on the effects the surrounding environment has on the isolation of APO. As well as studying the effect animals may have at each sample location, the collation of all inhibition seen at the three differing locations, could allow determination if farmland is more likely to exhibit higher amounts of APO than forest locations, due to the presence of more human and animal activity. However, there was not a significant increase in percentage of antimicrobial activity presented by Dawes Farm APO, compared to the percentage of total APO presenting inhibition from both Friston and New Forest. In the starvation method, only 20.9% of all APO isolated from Dawes Farm displayed inhibitory ability, much less than that seen by the APO tested from both Friston (30.4%) and New Forest (35.3%). Although nearly double the amount of farm APO presented inhibition in the perpendicular screening method, compared to the percentage of APO isolated from either of the two forests sampled; 30.2% of APO isolated from Dawes Farm

presented inhibitory activity to at least one of the five test bacteria, compared to that of 15.9% and 14.7% of the total APO isolated from Friston Forest and New Forest APO respectively, meaning conclusions cannot be drawn regarding the effect of animals on the microbiome. Most recent studies however only detail the antibiotic resistance conferred by pathogenic organisms within farmland sourced soil, rather that the ability for the isolation of APO from these farm locations (Wellington *et al.,* 2013; O'Neill, 2016; Wang *et al.,* 2016).

The cultivation and identification of potentially pathogenic bacteria was also carried out for further analysis and characterisation of the microbiome, of the differing locations. Large amounts of known soil microorganisms were prevalent in cultivation, including isolates belonging to the *Bacillus* spp., *Acinetobacter* spp., and *Kurthia* spp., as well as the isolation of organisms displaying similar characteristics to that of the *Streptomyces* spp. (see sections 3.3, 4.3 and 5.3). In total, 77 and 55 organisms were isolated from the New Forest and Friston Forest, with one (1.3%) and two (3.6%) organisms identified as belonging to the *Salmonella* spp., from these locations respectively. Organisms identified as *Streptomyces* were also isolated from these locations, with 11 (14.3%), identified from the New Forest, and two (3.6%), identified from the Friston Forest samples (See sections 3.3 and 4.3). Due to the location of *Streptomyces* from the wet soil samples, the testing of these isolates against test bacteria using the methods presented in this study, could be highlighted as further work, and would increase the amounts of isolates screened and the likelihood of locating novel antimicrobial compounds. However, as discussed in section 5.3, no inhibition was seen by the 11 (12.2%) *Streptomyces* isolates from Dawes Farm, that were tested further using the perpendicular screening method, meaning this further screening of *Streptomyces* isolates, from the wet soil samples, may be determined as unnecessary.

Further pathogenic organisms were also isolated from Dawes Farm, with 90 organisms isolated altogether, including those identified as *E. coli* (4.4%) and belonging to the *Klebsiella* spp. (4.4%), as well a large amount identified as belonging to the *Bacillus* spp. (28.9%), as discussed in section 5.3. However, overall a lack of organisms, of which were previously selected (see section 2.2.2.2), were isolated, from the three locations sampled, which may detail either the lack of these organisms in these environments, or that the organisms themselves are hard to culture. Further sampling however, would allow further characterisation of the sample locations, allowing conclusions to be drawn regarding the effects extended human, and animal activity, has on the soil microbiome, and whether this bares correlation to the presence of APO. As well as this, the majority of these organisms, were identified using biochemical tests only, with few being tested using API 20E. Other future work would include further biochemical tests, such as gelatin and carbohydrate utilisation, and genome sequencing to confirm the identification of these isolates, not used in the present study due to the high cost of sequencing, as well as time constraints. This confirmation would also allow the use of antibiograms to provide sensitivity results, and resistance profiles for

these pathogenic organisms, further allowing correlation to the activity of any APO isolated from the same sites. It should also be noted that the identification of these soil organisms is less specific than that seen for clinical isolates, due to the specificity of biochemical testing for the determination of well characterised clinical organisms, the vast amount of soil microorganisms, as well as the lesser known information on the characteristics of these soil organisms (Mazel and Davies, 1999; Abiola and Oyetayo, 2016).

Future work

The current study has demonstrated the ability to isolate APO from the New Forest, Friston Forest, and Dawes Farm, with activity against both sensitive and clinical strains of test bacteria. Several areas however, have been highlighted in need of further research, with the potential for further development of the study in various areas. In hindsight, it may be favourable to carry out future work on single locations, to allow a more in-depth analysis of the microbiome of the particular area being studied, as well as permitting time for repeat sampling at differing time points for further comparative analysis of the changes in the microbiome. This may also include further assessment of the soil biochemistry, in relation to the presence of APO, in partnership with the Forestry Commission.

Other future work focussing on the isolates cultivated in the present study includes the continuing of the identification of *Streptomyces* using PCR, as well as the potential use of genomic sequencing of both the APO and supposed pathogenic bacteria, to allow conclusive identification and correlation, between the isolated strains. The completion of testing of all APO from the three locations, as well as carrying out further repeats of both perpendicular screening, and the starvation method, including tests against clinical organisms, would also be favourable to be carried out, to allow further conclusions to be drawn, as well as determining if the stress induced environment, used in the starvation method, encourages bacterial organisms to inhibit clinical, as well as sensitive strains. This further testing may also include the addition of isolates discussed above, as well as the inclusion of both multi-drug and pan-drug resistant organisms, to further determine the inhibitory ability of the compounds produced. As well as this, the collection of further samples, from the three locations used in this study, at differing times of the year, would also allow the effects of seasonal changes on the microbiome, to be studied. The completion of this further work would also allow additional statistical and comparative analysis to be carried out on the data produced from the three locations, and for the determination of the significance of the location on the likelihood for the isolation of APO.

Further work could also include the extraction and purification of inhibitory compounds, allowing pure antibiotic substances to be obtained, which will likely increase the inhibitory action and inhibition zones seen, as well as allowing the active structures to be determined using such methods as NMR and/or mass spectrometry (Bizuye *et al.,* 2013). This was trialled in the pilot study discussed in sections 2.2.5 and 3.2.3.3 using the supernatant produced by isolate 26 in the starvation method for chemical analysis, involving the use of a TLC bioassay, as well as NMR. However, as discussed in section 3.3, the true structure of the three supposed antibacterial compounds could not be determined due to time constraints in the current project, and it was highlighted that further methods, including mass spectrometry, would aid in the determination of structures. Continuation of this pilot study, using other supernatants produced by inhibitory APO in the starvation method, as well as carrying out the additional methods discussed, would allow the elucidation and confirmation of compound structures, and determination of those that are novel.

The present study is thought to be the first study isolating APO from Friston Forest, New Forest and Dawes Farm, however it may be likely that any inhibition seen is by compounds that have already been discovered. It is also known that around 99% of soil microorganisms, cannot be grown in laboratory conditions and differing techniques are required for the cultivation of these organisms, such as those discussed by Ling and colleagues (2015). This may indicate that the APO identified in this study have already been isolated and examined (Mazel and Davies, 1999). This also highlights the need for differing methods, such as those discussed by several other studies, that focus on altering metabolic pathways, in already known organisms, to induce the production of inhibitory secondary metabolites from the primary metabolism (Bekker *et al.,* 2015). This is known as metabolic engineering, which considers the connections between gene expression and secondary metabolite production allowing control mechanisms within the cell to be understood and manipulated (see figure 3.8) (Rokem *et al.,* 2007). This has allowed the improvement of cellular activities, as well as increased efficiency, and yield of secondary metabolites of interest from certain organisms (Rokem *et al.,* 2007; Bekker *et al.,* 2015). This engineering may seem favourable over the starvation method, as this altering of biosynthetic pathways, also allows the optimisation of environmental and growth conditions, for the production of antimicrobial compounds of interest. Whereas the starvation method, used in this study, relies on the creation of generalised stress conditions, in the hope it causes a stringent response in APO, leading to a production of inhibitory metabolites. However, genetic engineering requires a vast amount of previous knowledge on the biochemistry, physiology and genetics of the APO of interest, which may present problems due to the vast amounts of differing, potentially rare, soil organisms isolated (Rokem *et al.,* 2007). In the future, this molecular work could be carried out on the confirmed *Streptomyces* strains, due to the use of the StreptomeDB database, which contains information for over 2500 different strains of *Streptomyces* spp. and their products providing sufficient information for the determination of

engineering strategies for the production of secondary metabolites of interest (Lucas *et al.,* 2013; Klementz *et al.,* 2016).

However as discussed previously, one novel antibiotic is not enough to overcome the AMR crisis. The research and development, of novel antimicrobials is an ongoing process, necessary to ensure pathogenic infections remain treatable (Laxminarayan *et al.,* 2013). Several objectives have been put into place, including antibiotic stewardship, conservation and surveillance, as well as increasing global awareness, to maintain sensitivity in pathogenic bacteria, allowing antimicrobial treatment to remain effective, and actively combat antibiotic resistance (O'Neill, 2016). Nevertheless, this study forwards the research of the isolation and production of novel antimicrobials necessary as part of the fight against AMR.

7.0 Conclusion

In conclusion, this study confirms that APO can in fact be isolated from both Friston (a modern forest), New Forest (an ancient forest), and Dawes Farm (a working farmland). Inhibitory activity was seen from all locations, against at least one of the clinical strains of test bacteria. The results seen in the present study highlight the potential of soil microorganisms, for the isolation of potentially novel antimicrobial compounds, which could largely contribute to the fight against AMR, as a promising source of new antibiotics, that are desperately needed.

8.0 References

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9.0 Appendix

Table A1: Geographic coordinates, and area description of the soil samples taken from the New Forest, in November 2016.

Soil Sample Number	Description of area	Latitude	Longitude
1	Hatchpond car park by tree	50.812026	-1.477028
2	Hatchpond car park by tree	50.812026	-1.477028
3	Hatchpond car by pond	50.812026	-1.477028
4	Hatchpond car by pond	50.812026	-1.477028
5	Balmer lawn by river	50.826604	-1.540752
6	Balmer lawn by river	50.826604	-1.540752
7	Balmer lawn under tree	50.826604	-1.540752
8	Balmer lawn under tree	50.826604	-1.540752
9	Setthorns by tree	50.796523	-1.616355
10	Setthorns by tree	50.796523	-1.616355
11	Setthorns on path	50.79644.0	-1.616447
12	Setthorns on path	50.79644.0	-1.616447
13	Setthorns by tree and horse faeces.	50.805486	-0.1641835
14	Setthorns by tree and horse faeces.	50.805486	-0.1641835
15	Wilverley Scout campsite by cow faeces.	50.806488	-1.643814
16	Wilverley Scout campsite by cow faeces.	50.806488	-1.643814
17	Wilverley Scout campsite by tree	50.806887	-1.643917
18	Wilverley Scout campsite by tree	50.806887	-1.643917
19	Longbeech by tree	50.913909	-1.642163
20	Longbeech by tree	50.913909	-1.642163
21	Longbeech on grass	50.913874	-1.642141
22	Longbeech on grass	50.913874	-1.642141
23	Shavewood by car park	50.908014	-1.58358
24	Shavewood by car park	50.908014	-1.58358
25	Shavewood by tree	50.907906	-1.584144
26	Shavewood by tree	50.907906	-1.584144
27	Bolderwood arboretum by tree	50.907906	-1.584144
28	Bolderwood arboretum by tree	50.907906	-1.584144
29	Bolderwood arboretum near road (lot of moss)	50.867698	-1.654995
30	Bolderwood arboretum by tree	50.867698	-1.654995
31	Knightwood oak by car park near tree	50.856119	-1.626748
32	Knightwood oak by car park near tree	50.856119	-1.626748
33	Knightwood oak open	50.856119	-1.626748
34	Knightwood oak open	50.856119	-1.626748
35	Bolton bench near tree	50.87288.0	-1.568326
36	Bolton bench near tree	50.87288.0	-1.568326
37	Bolton bench near faeces in open	50.872728	-1.568315
38	Bolton bench near faeces in open	50.872728	-1.568315
39	Shatterford near tree	50.855284	-1.506576
40	Shatterford near tree	50.855284	-1.506576
41	Shatterford open	50.85587.0	-1.506766
42	Shatterford open	50.85587.0	-1.506766

Table A2: The catalase and Gram stain result of all 34 APO, isolated using potato starch agar from the New Forest soil samples.

Isolate Number	Sample location	Catalase test	Gram Stain
1	7	+VE	Gram-positive rods with spores.
2	7	+VE	Gram-positive rods.
3	7	+VE	Gram-positive rods with spores.
4	11	+VE	Gram-positive rods
5	11	+VE	Gram-positive rods
6	11	+VE	Gram-negative rods
7	16	+VE	Gram-positive rods
8	16	+VE	Gram-positive rods
9	16	+VE	Gram-positive rods
10	16	+VE	Gram-positive rods
11	16	+VE	Gram-positive rods with spores.
12	19	+VE	Gram-positive rods
13	20	+VE	Gram-positive rods
14	20	+VE	Gram-positive rods with spores.
15	22	+VE	Gram-positive rods.
16	22	-VE	Gram-negative rods.
17	22	+VE	Gram-positive rods.
18	22	+VE	Gram-positive rods.
19	22	+VE	Gram-positive rods.
20	22	-VE	Long Gram-positive rods.
21	22	+VE	Gram-Negative rods.
22	22	+VE	Gram-positive rods.
23	24	+VE	Gram-negative rods.
24	27	+VE	Gram-negative rods
25	27	+VE	Gram-positive rods.
26	36	-VE	Gram-negative rods.
27	36	+VE	Gram-negative rods.
28	37	+VE	Gram-negative rods.
29	37	+VE	Gram-positive rods.
30	39	+VE	Gram-positive rods.
31	39	+VE	Small Gram-positive rods.
32	39	+VE	Gram-positive rods with spores in chains.
33	39	+VE	Gram-negative rods.
34	39	+VE	Gram-positive rods.

Table A3: Sample location and information of soil samples collected from Friston Forest, in April 2017. Note that the location results were converted to latitude and longitude, from the original results, taken using grid reference coordinates.

Sample number	Latitude	Longitude	Description of area
1	50.782627	0.153862	Outer forest
2	50.782627	0.153862	Outer forest
3	50.782986	0.160057	Roadside
4	50.782986	0.160057	Within forest
5	50.777509	0.193268	Roadside
6	50.777509	0.193268	Within forest
7	~50.777509	~0.193268	Roadside
8	~50.777509	~0.193268	Within forest
9	50.784134	0.192958	Picnic area
10	~50.784134	~0.192958	Within Forest
11	~50.784134	~0.192958	Within Forest
12	~50.784134	~0.192958	Near tree (acidic)
13	~50.784134	~0.192958	Near tree (acidic)
14	~50.784134	~0.192958	Within Forest
15	50.784769	0.200585	Within Forest
16	50.784769	0.200585	Within Forest
17	50.776317	0.171040	Within Forest
18	50.776317	0.171040	Within Forest
19	50.776073	0.189068	Within forest
20	50.776073	0.189068	Near cycle path and Rabbit faeces.

Table A4: Catalase and Gram stain of the 31 APO isolated from Friston Forest using oat agar. (S) indicates the presence of a potential *Streptomyces* spp., due to morphology and/or Gram stain result, which were selected for further identification using DNA extraction, PCR and Gel electrophoresis.

Isolate number	Sample location	Catalase test	Gram stain
35	1	+VE	Long Gram-positive rods (S)
36	1	+VE	Gram-positive rods with spores
37	4	+VE	Long Gram-positive rods (S)
38	4	+VE	Gram-negative rods
39	5	-VE	Hollow Gram-positive rods
40	5	+VE	Gram-positive rods
41	6	+VE	Gram-positive rods
42	6	-VE	Long Gram-positive rods (S)
43	6	-VE	Long Gram-positive rods (S)
44	8	+VE	Gram-positive rods with spores
45	8	+VE	Long Gram-positive rods (S)
46	8	+VE	Long Gram-positive rods (S)
47	8	+VE	Gram-positive rods
48	8	+VE	Gram-positive rods
49	9	+VE	Long Gram-positive rods (S)
50	9	+VE	Gram-positive rods with spores
51	9	+VE	Gram-positive rods with spores
52	10	+VE	Gram-positive rods with spores
53	13	+VE	Long Gram-positive rods (S)
54	14	+VE	Gram-positive rods with spores
55	14	+VE	Gram-positive rods
56	14	+VE	Gram-positive rods with spores
57	16	+VE	Gram-negative rods
58	17	+VE	Long Gram-positive rods (S)
59	17	+VE	Long Gram-positive rods (S)
60	17	+VE	Long Gram-positive rods (S)
61	18	-VE	Gram-positive rods with spores
62	18	+VE	Long Gram-positive rods (S)
63	19	+VE	Gram-positive rods with spores
64	20	+VE	Long Gram-positive rods (S)
65	20	+VE	Gram-positive rods

Table A5: Catalase and Gram stains results of the 38 APO isolated from Friston Forest using potato starch agar. (S) indicates the presence of a potential *Streptomyces* spp. due to morphology and/or Gram stain result, and those selected for further identification using DNA extraction, PCR and Gel electrophoresis.

Isolate number	Sample location	Catalase test	Gram stain
66	2	+VE	Gram-positive rods
67	2	+VE	Long Gram-positive rods (S)
68	2	+VE	Long Gram-positive rods (S)
69	2	+VE	Gram-positive rods
70	3	+VE	Gram-positive rods
71	3	+VE	Gram-positive rods with spores
72	4	+VE	Long Gram-positive rods (S)
73	4	-VE	Gram-positive rods
74	4	+VE	Gram-positive rods
75	4	+VE	Long Gram-positive rods (S)
76	5	+VE	Long Gram-positive rods with (S)
77	5	+VE	Gram-positive rods
78	5	+VE	Gram-negative rods
79	6	+VE	Long Gram-positive rods (S)
80	6	+VE	Gram-positive rods
81	6	+VE	Long Gram-positive rods (S)
82	7	+VE	Gram-positive rods with spores
83	7	+VE	Gram-positive rods with spores
84	8	+VE	Long Gram-positive rods (S)
85	8	+VE	Long Gram-positive rods (S)
86	8	+VE	Long Gram-positive rods (S)
87	8	+VE	Gram-positive rods
88	10	+VE	Long Gram-positive rods (S)
89	10	+VE	Gram-positive rods
90	10	+VE	Gram-positive rods
91	10	+VE	Long Gram-positive rods (S)
92	13	+VE	Long Gram-positive rods (S)
93	13	+VE	Gram-positive rods with spores
94	13	+VE	Gram-positive rods
95	13	+VE	Long Gram-positive rods (S)
96	14	+VE	Long Gram-positive rods (S)
97	16	+VE	Long Gram-positive rods (S)
98	18	+VE	Long Gram-positive rods (S)
99	19	+VE	Gram-positive rods
100	19	+VE	Gram-negative rods (S)
101	19	+VE	Gram-positive rods
102	20	+VE	Long Gram-positive rods (S)
103	20	+VE	Long Gram-positive rods (S)

Table A6: The DNA concentration and purity results of the 13 isolates, in duplicate, with similar morphology and/or Gram stain to *Streptomyce*s spp. from Friston Forest initially cultivated using oat agar. Also details the identification of *Streptomyces* spp. within these isolates using the DNA extracted results shown.

Oat agar Isolate number	DNA concentration (ng/ µl)	A260/A280	A260/A230	*Streptomyces* spp.? (+/-)
35	167	1.988	1.777	+VE
35	166	1.953	1.431	+VE
37	-	-	-	-
37	-	-	-	-
42	115	1.769	1.075	+VE
42	169	1.742	1.174	+VE
43	43	1.706	0.606	+VE
43	51	1.686	0.586	+VE
45	21	1.798	0.934	+VE
45	12	1.347	0.539	+VE
46	74	1.682	1.156	+VE
46	52	1.905	1.677	+VE
49	109	1.758	1.313	+VE
49	188	1.725	1.190	+VE
53	30	1.606	0.487	-
53	48	1.553	0.738	-
58	-	-	-	-
58	-	-	-	-
59	193	1.804	1.451	+VE
59	63	1.818	1.400	+VE
60	233	1.739	0.975	-
60	211	1.744	1.185	-
62	-	-	-	-
62	-	-	-	-
64	-	-	-	-
64	-	-	-	-

Table A7: The purity and concentration of DNA extracted from 20 APO isolated from Friston Forest using potato starch agar, selected due to similar morphology and/or Gram stain to that of the *Streptomyces* spp. Also details the identification of *Streptomyces* spp. confirmed by use of the DNA shown, PCR and Gel electrophoresis.

Potato agar Isolate number	DNA concentration (ng/ µl)	A260/A280	A260/A230	*Streptomyces* spp.? (+/-)
67	102	1.729	1.259	-VE
67	118	1.873	1.356	-VE
68	333	1.673	0.856	-VE
68	496	1.710	0.934	-VE
72	329	1.848	1.437	+VE
72	306	1.877	1.286	+VE
75	119	1.322	0.254	-VE
75	120	1.319	0.254	-VE
76	12	1.732	1.522	+VE
76	34	1.802	1.877	+VE
79	-	-	-	-
79	-	-	-	-
81	118	1.229	0.263	+VE
81	113	1.215	0.259	+VE
84	212	1.514	0.400	+VE
84	160	1.416	0.406	+VE
85	-	-	-	-
85	-	-	-	-
86	-	-	-	-
86	-	-	-	-
88	131	1.323	0.287	-VE
88	126	1.273	0.294	-VE
91	145	1.368	0.302	+VE
91	145	1.355	0.298	+VE
92	31	1.373	0.282	+VE
92	32	1.330	0.285	+VE
95	25	1.380	0.296	+VE
95	21	1.359	0.275	+VE
96	595	1.740	1.068	+VE
96	588	1.740	1.083	+VE
97	10	1.944	0.901	+VE
97	18	1.897	0.664	+VE
98	41	2.158	1.343	+VE
98	38	2.188	1.221	+VE
100	31	1.374	0.313	+VE
100	32	1.491	0.347	+VE
102	39	1.501	0.406	+VE
102	42	1.547	0.433	+VE
103	189	1.835	0.871	-
103	183	1.830	0.906	-

Table A8: The sample locations at Dawes Farm, where soil was aseptically sampled in May 2017. PP was the name of one sample location, due to it being sampled from faecal matter, within the cow field. One sample location was noted for samples 13-22 (and PP), due to the collection of the samples in differing areas within in the same field.

Soil sample number	Location information	Longitude	Latitude
1	Cow field	-0.376818	51.108043
2	Cow field	-0.376818	51.108043
3	Cow field	-0.376818	51.108043
4	Cow field	-0.376818	51.108043
5	Cow field- near tree	-0.377956	51.106778
6	Cow field- near tree	-0.377956	51.106778
7	Cow field – near water trough.	-0.375476	51.106483
8	Cow field – near water trough.	-0.375476	51.106483
9	Cow field – near different water trough.	-0.374799	51.107715
10	Cow field – near different water trough.	-0.374799	51.107715
11	Footpath between fields.	-0.374501	51.107662
12	Footpath between fields.	-0.374501	51.107662
13	Cow field – with cow's present.	~ -0.375397	~ 51.109048
14	Cow field – with cow's present.	~ -0.375397	~ 51.109048
15	Cow field – with cow's present.	~ -0.375397	~ 51.109048
16	Cow field – with cow's present.	~ -0.375397	~ 51.109048
17	Cow field – with cow's present.	~ -0.375397	~ 51.109048
18	Cow field – with cow's present.	~ -0.375397	~ 51.109048
19	Cow field – with cow's present.	~ -0.375397	~ 51.109048
21	Cow field – with cow's present near tree.	~ -0.375397	~ 51.109048
22	Cow field – with cow's present near tree.	~ -0.375397	~ 51.109048
Faecal matter (PP)	Cow field – with cow's present.	~ -0.375397	~ 51.109048

Table A9: Catalase and Gram strains of the 26 APO isolated from Dawes Farm using oat agar. (S) indicates the presence of a potential *Streptomyces* spp., due to morphology and/or Gram stain result, and hence those selected for further identification using DNA extraction, PCR and Gel electrophoresis.

Isolate number	Sample location	Catalase test	Gram Stain
104	2	+VE	Gram-negative rods
105	4	+VE	Gram-negative rods
106	5	+VE	Gram-positive cocci
107	6	+VE	Long Gram-positive rods (S)
108	7	+VE	Gram-positive rods and spores
109	9	+VE	Long Gram-positive rods (S)
110	9	-VE	Gram-negative rods
111	9	+VE	Gram-negative rods
112	9	+VE	Gram-negative rods.
113	10	+VE	Gram-positive rods.
114	10	+VE	Gram-positive rods.
115	11	+VE	Long Gram-positive rods (S)
116	11	+VE	Gram-positive rods.
117	12	+VE	Small Gram-negative rods.
118	12	+VE	Long Gram-positive rods (S)
119	12	+VE	Gram-positive rods with spores.
120	13	+VE	Gram-positive rods.
121	13	+VE	Long Gram-positive rods (S)
122	14	+VE	Gram-positive rods.
123	16	+VE	Long Gram-positive rods (S)
124	18	+VE	Long Gram-positive rods (S)
125	19	+VE	Long Gram-positive rods (S)
126	21	+VE	Long Gram-positive rods (S)
127	22	+VE	Long Gram-positive rods (S)
128	22	+VE	Long Gram-positive rods (S)
129	PP	+VE	Gram-positive cocci.

Table A10: Catalase and Gram stains of the 17 APO isolated from Dawes Farm using potato starch agar. (S) indicates the presence of a potential *Streptomyces* spp. due to morphology and/or Gram stain, and hence those selected for further identification using DNA extraction, PCR and Gel electrophoresis.

Isolate number	Sample locations	Catalase test	Gram stain
130	2	+VE	Gram-positive rods
131	4	+VE	Gram-negative rods
132	4	+VE	Gram-negative rods
133	6	+VE	Long Gram-positive rods (S)
134	6	+VE	Long Gram-positive rods (S)
135	6	+VE	Gram-positive rods with spores
136	7	+VE	Gram-negative rods
137	9	+VE	Long Gram-positive rods (S)
138	10	+VE	Gram-positive rods with spores
139	11	+VE	Long Gram-positive rods (S)
140	12	+VE	Long Gram-positive rods (S)
141	12	+VE	Long Gram-positive rods (S)
142	14	+VE	Gram-positive rods with spores
143	18	+VE	Long Gram-positive rods (S)
144	18	+VE	Long Gram-positive rods (S)
145	19	+VE	Gram-positive rods with spores
146	22	+VE	Long Gram-positive rods (S)

Table A11: The concentration and purity of the DNA extracted in duplicate, from the 11 Dawes Farm APO originally isolated using oat agar, with similar characteristics to the *Streptomyces* genus. Isolates with either low concentration of DNA and or poor quality detailed as inconclusive (-).

Isolate Number	Concentration (ng/ µl)	A260/A280	A260/A230
107	-	-	-
107	-	-	-
109	168	2.182	2.100
109	167	2.227	2.400
115	119	1.991	1.008
115	142	1.844	1.029
118	-	-	-
118	-	-	-
121	-	-	-
121	-	-	-
123	184	1.353	0.320
123	163	1.304	0.392
124	-	-	-
124	-	-	-
125	-	-	-
125	-	-	-
126	-	-	-
126	-	-	-
127	-	-	-
127	-	-	-
128	-	-	-
128	-	-	-

Table A12: The DNA concentration and purity results of isolates, with characteristics similar to that of the *Streptomyces* genus, and initially cultivated from Dawes Farm soil samples, using potato starch agar.

Isolate number	Concentration (ng/ µl)	A260/A280	A260/A230
133	413	1.721	1.144
133	361	1.719	1.003
134	444	1.776	1.251
134	301	1.824	1.400
137	180	1.667	0.811
137	155	1.685	0.876
139	42	1.673	2.154
139	62	1.588	1.148
140	62	2.196	4.667
140	65	1.892	1.697
141	141	2.104	1.880
141	150	2.113	2.000
143	93	2.255	2.290
143	83	2.096	1.976
144	396	1.616	0.747
144	368	1.614	0.732
146	104	1.733	2.925
146	133	1.705	1.198

Table A13: The susceptibility profiles of MRSA, *E. coli*, and *P. mirabilis*, that were clinically isolated, and used for further APO testing in the perpendicular screening method. Susceptibility was determined using EUCAST clinical breakpoint tables for antimicrobial susceptibility (EUCAST, 2017). Susceptibility is indicated as Sensitive (S), Intermediate (I) or Resistant (R). Antibiotics used were as follows; Ciprofloxacin (5 µg) (CIP5), Erythromycin (5 µg) (E5), Oxytetracycline (30 µg) (OT30), Gentamycin (10 µg) (CN10), Cefoxitin (30 µg) (FOX30), Amoxicillin and Clavulanic acid (30 µg) (AMC30), Imipenem (10 µg) (IPM10), Cefotaxime (5 µg) (CTX5). Oxytetracycline susceptibility profiles were not found, but were kept in tables for reference.

Methicillin- resistant *S. aureus* sensitivity			***E. coli* sensitivity**			***P. mirabilis* sensitivity**		
Antibiotic disc:	**Diameter of inhibition (mm)**	**Susceptibility**	**Antibiotic**	**Diameter of inhibition (mm)**	**Susceptibility**	**Antibiotic**	**Diameter of inhibition (mm)**	**Susceptibility**
CIP5	25	S	AMC30	13	R	AMC30	28	S
E5	10	R	IPM10	30	S	IPM10	30	S
OT30	8	-	CTX5	25	I	CTX5	31	S
CN10	20	S	CIP5	30	S	CIP5	32	S
FOX30	25	S	CN10	6	R	CN10	21	S

Acquisition Time (sec)	4.9633	**Comment**	5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079			**D**	0.06	**D1**	1
DE	12.93771	**DS**	2	**Date**	18 Jul 2017 15:27:28			**Date Stamp**	18 Jul 2017 15:27:28
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PROBHD	<5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079 >			**PULPROG**	<zg30>	**Points Count**	65536	**Pulse Sequence**	zg30
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SI	65536	**SSB**	0	**SW(cyclical) (Hz)**	6602.11	**SWH**	6602.11267605634		
Solvent	CHLOROFORM-d			**Spectrum Offset (Hz)**	2986.1274	**Spectrum Type**	standard	**Sweep Width (Hz)**	6602.01
TD	65536	**TD0**	1	**TE**	297.96	**Temperature (degree C)**	24.960	**UNC1**	<1H>
WDW	1								

^{1}H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 0.80 - 0.86 (m, 246 H) 0.90 - 0.96 (m, 73 H) 1.38 - 1.45 (m, 77 H) 1.60 - 1.65 (m, 32 H) 1.65 - 1.71 (m, 28 H) 1.99 - 2.03 (m, 14 H) 2.13 (s, 2 H) 2.22 (t, *J*=7.64 Hz, 10 H) 2.27 - 2.36 (m, 26 H) 2.37 - 2.44 (m, 7 H) 2.60 (t, *J*=1.00 Hz, 8 H) 2.80 (t, *J*=7.47 Hz, 6 H) 2.83 (s, 6 H) 3.64 - 3.67 (m, 8 H) 3.76 (s, 2 H) 4.09 (br quin, *J*=6.80 Hz, 10 H) 4.12 - 4.16 (m, 4 H) 4.22 (qd, *J*=11.09, 6.00 Hz, 15 H) 5.35 (t, *J*=4.91 Hz, 6 H) 6.51 (s, 2 H) 6.98 (s, 3 H) 7.08 (s, 2 H) 7.13 (dd, *J*=8.60, 2.51 Hz, 6 H) 7.34 - 7.37 (m, 5 H) 7.43 (s, 2 H) 7.51 - 7.55 (m, 13 H) 7.71 (dd, *J*=1.00 Hz, 8 H) 8.10 (s, 2 H) 9.76 (s, 2 H)

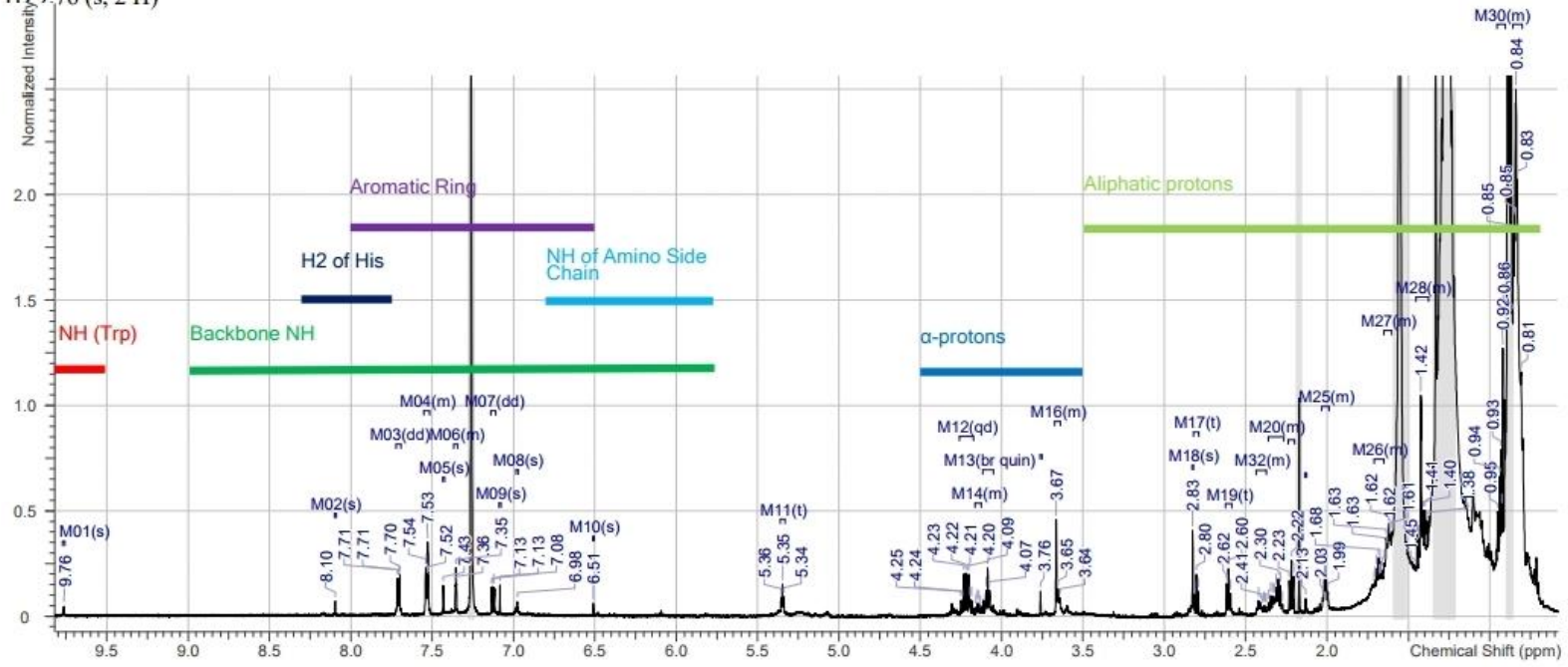

Figure A1: The NMR results of antibacterial compound 1, extracted using the solvent dichloromethane, with an original Rf value of 1 (see table 3.8). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

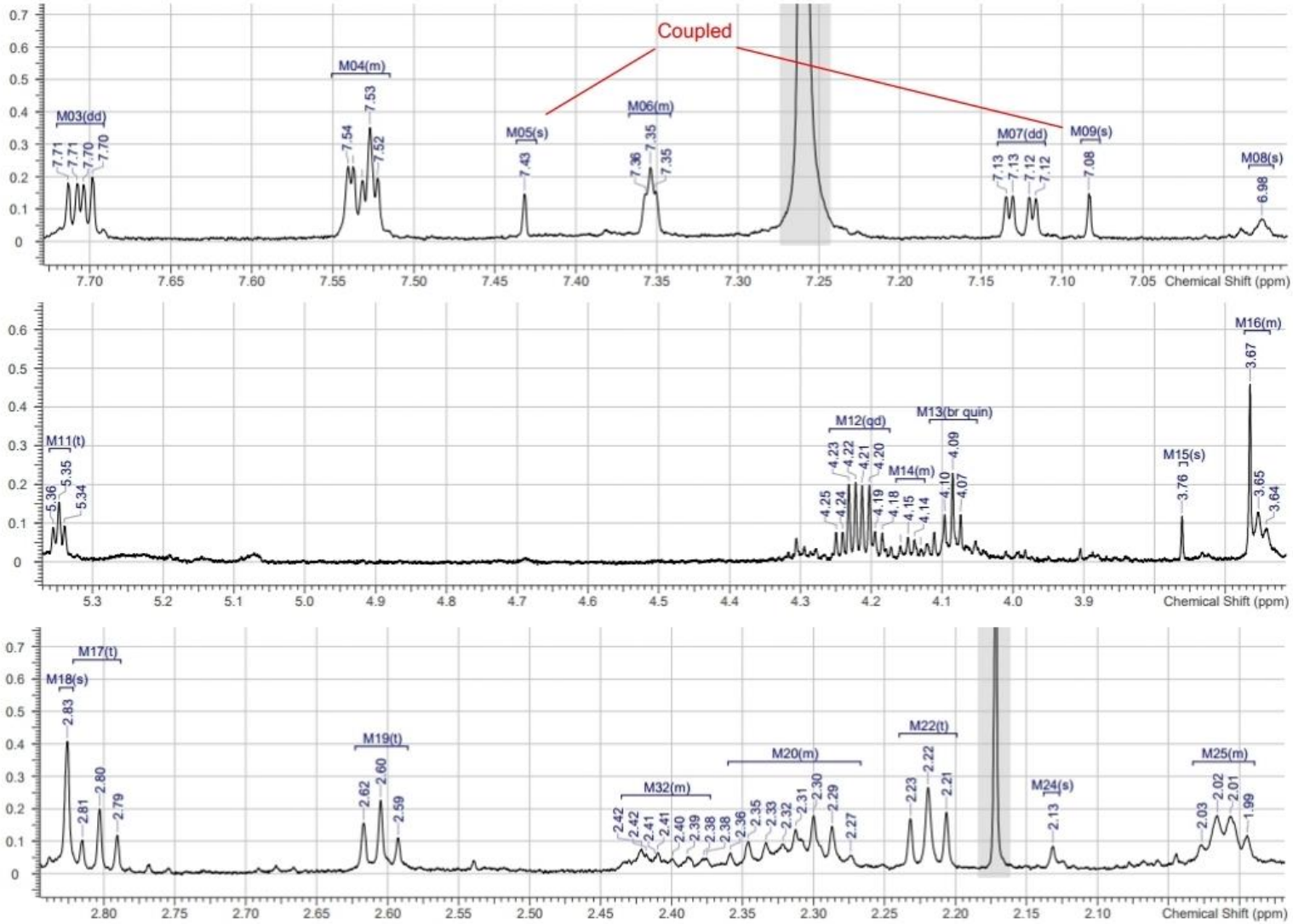

Figure A2: Supplementary NMR results of antibacterial compound 1, extracted using the solvent dichloromethane, with an original Rf value of 1 (Table 3.8). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

Acquisition Time (sec)	4.9633	**Comment**	5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079			**D**	0.06	**D1**	1
DE	12.93771	**DS**	2	**Date**	20 Jul 2017 17:58:24			**Date Stamp**	20 Jul 2017 17:58:24
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INSTRUM	<av600>	**LB**	0.2	**NS**	256	**Nucleus**	1H	**Number of Transients**	256
Origin	av600	**Original Points Count**	32768	**Owner**	nmrsu	**PC**	1		
PROBHD	<5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079 >			**PULPROG**	<zg30>	**Points Count**	65536	**Pulse Sequence**	zg30
Receiver Gain	406.00	**SF**	600.130014354653			**SFO1**	600.13300065		
SI	65536	**SSB**	0	**SW(cyclical) (Hz)**	6602.11	**SWH**	6602.11267605634		
Solvent	CHLOROFORM-d			**Spectrum Offset (Hz)**	2986.2957	**Spectrum Type**	standard	**Sweep Width (Hz)**	6602.01
TD	65536	**TD0**	1	**TE**	297.96	**Temperature (degree C)**	24.960	**UNC1**	<1H>
WDW	1								

^{1}H NMR (600 MHz, CHLOROFORM-*d*) δ ppm 0.90 - 0.96 (m, 227 H) 0.98 - 1.07 (m, 248 H) 1.16 (br s, 236 H) 1.39 (q, *J*=1.00 Hz, 28 H) 1.47 (d, *J*=6.81 Hz, 37 H) 1.85 - 1.99 (m, 98 H) 1.99 - 2.08 (m, 116 H) 2.11 (s, 8 H) 2.20 - 2.24 (m, 21 H) 2.35 (t, *J*=1.00 Hz, 64 H) 2.37 - 2.47 (m, 64 H) 2.59 (s, 4 H) 2.63 - 2.68 (m, 15 H) 2.80 (br d, *J*=1.00 Hz, 9 H) 2.84 - 2.93 (m, 14 H) 3.02 - 3.15 (m, 15 H) 3.44 - 3.57 (m, 202 H) 3.58 - 3.75 (m, 29 H) 3.84 (br d, *J*=1.00 Hz, 7 H) 3.88 - 3.96 (m, 57 H) 4.02 (br d, *J*=11.82 Hz, 15 H) 4.06 - 4.12 (m, 42 H) 4.13 - 4.17 (m, 40 H) 4.18 - 4.23 (m, 32 H) 4.59 (br s, 5 H) 4.81 (dd, *J*=11.48, 2.92 Hz, 3 H) 4.93 (t, *J*=4.69 Hz, 3 H) 5.34 (br d, *J*=6.01 Hz, 7 H) 5.74 (br s, 25 H) 5.90 (br s, 23 H) 6.02 (br s, 18 H) 6.06 (br s, 9 H) 6.95 (s, 3 H) 7.08 (s, 4 H) 7.13 (d, *J*=7.32 Hz, 7 H) 7.19 - 7.24 (m, 26 H) 7.28 - 7.32 (m, 28 H) 7.43 (s, 5 H) 7.53 (dd, *J*=1.00 Hz, 4 H) 7.70 (dd, *J*=1.00 Hz, 3 H) 7.86 (d, *J*=3.08 Hz, 4 H) 8.18 (s, 3 H) 8.31 - 8.34 (m, 4 H) 10.07 (s, 3 H)

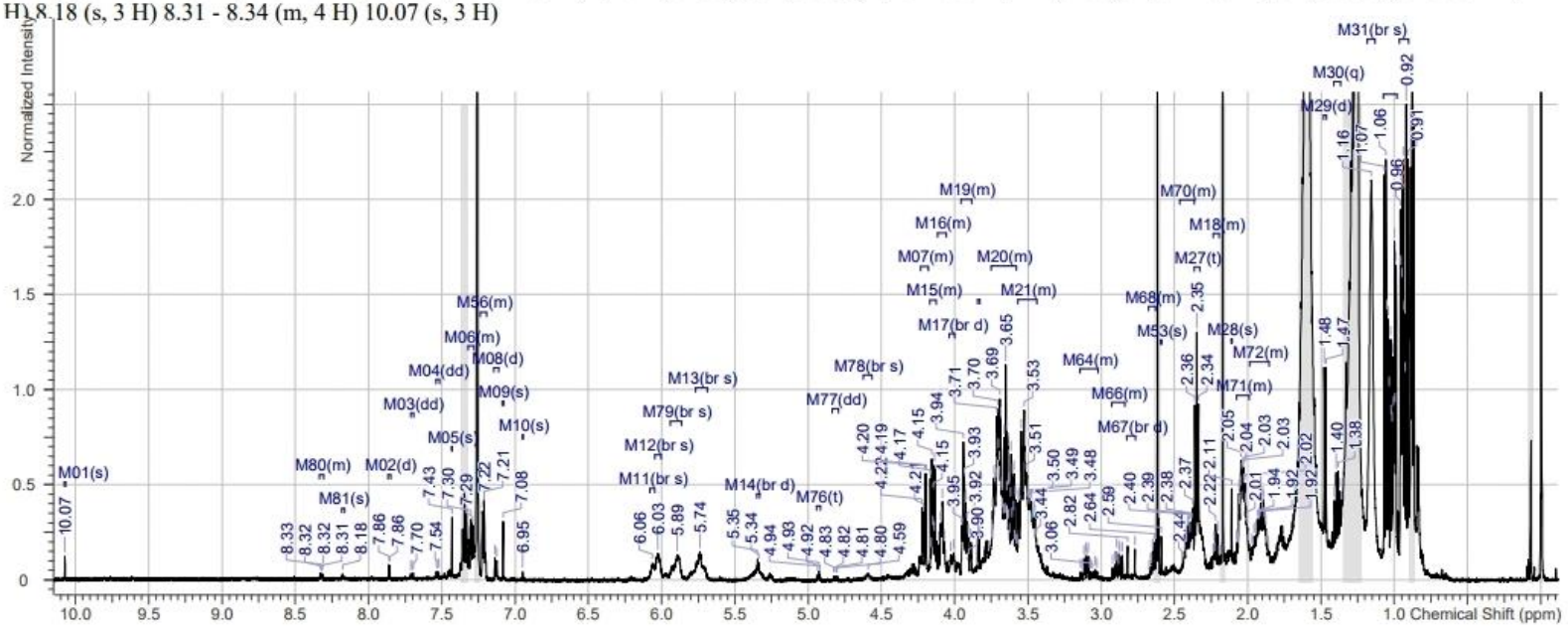

Figure A3: NMR data for compound 2, extracted using dichloromethane, with a resulting Rf value of 0.68 (Table 3.8). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

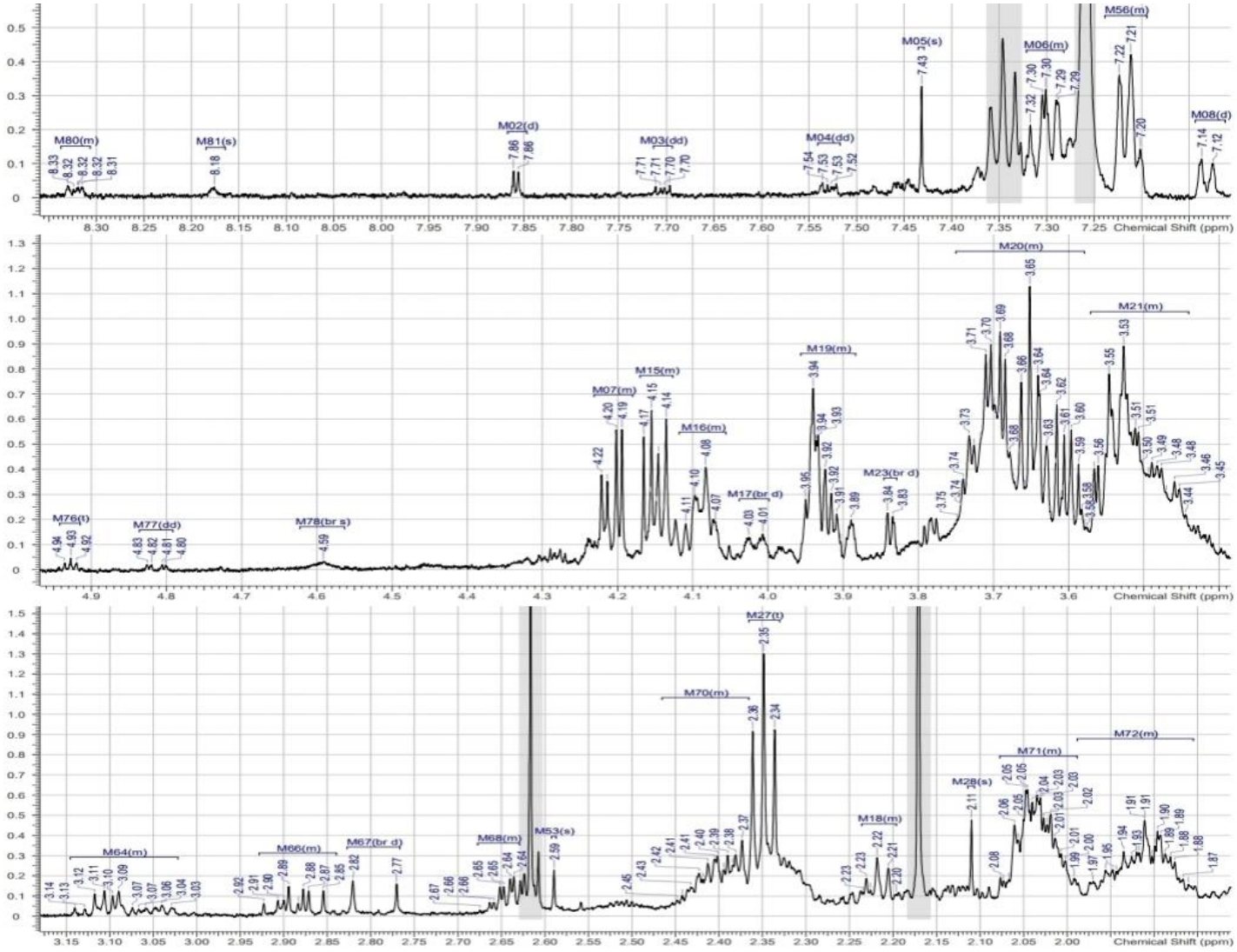

Figure A4: Supplementary NMR data for compound 2, extracted using dichloromethane, with a resulting Rf value of 0.68 (Table 3.8). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

Acquisition Time (sec)	4.9633	**Comment**	5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079			**D**	0.06	**D1**	1
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PROBHD	<5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079 >			**PULPROG**	<zg30>	**Points Count**	65536	**Pulse Sequence**	zg30
Receiver Gain	406.00	**SF**	600.129993496929			**SFO1**	600.13300065		
SI	65536	**SSB**	0	**SW(cyclical) (Hz)**	6602.11	**SWH**	6602.11267605634		
Solvent	METHANOL-d4			**Spectrum Offset (Hz)**	3007.1504	**Spectrum Type**	standard	**Sweep Width (Hz)**	6602.01
TD	65536	**TD0**	1	**TE**	297.96	**Temperature (degree C)**	24.960	**UNC1**	<1H>
WDW	1								

^{1}H NMR (600 MHz, METHANOL-d_4) δ ppm 0.97 (q, *J*=1.00 Hz, 6 H) 1.21 (br t, *J*=7.30 Hz, 2 H) 1.24 - 1.26 (m, 1 H) 1.51 - 1.58 (m, 5 H) 1.63 (br s, 7 H) 1.95 (br s, 5 H) 2.04 - 2.08 (m, 1 H) 2.22 (dt, *J*=1.00 Hz, 3 H) 2.73 (s, 1 H) 3.22 (dt, *J*=3.29, 1.63 Hz, 1 H) 3.36 - 3.36 (m, 1 H) 3.38 (s, 1 H) 3.45 (dt, *J*=1.00 Hz, 1 H) 3.57 (d, *J*=6.50 Hz, 1 H) 3.59 - 3.59 (m, 1 H) 3.60 (br s, 1 H) 3.61 (s, 1 H) 3.63 (d, *J*=0.88 Hz, 1 H) 3.64 (s, 1 H) 3.83 (td, *J*=5.26, 1.26 Hz, 1 H) 3.90 (s, 1 H) 4.05 (s, 1 H) 4.25 (ddd, *J*=5.64, 4.33, 1.11 Hz, 1 H) 4.36 - 4.40 (m, 1 H) 4.96 (br t, *J*=3.78 Hz, 1 H) 5.37 (ddd, *J*=5.74, 4.53, 0.91 Hz, 1 H) 6.76 (br d, *J*=10.07 Hz, 1 H) 7.25 (dd, *J*=1.00 Hz, 1 H) 7.47 - 7.49 (m, 1 H) 7.66 (dd, *J*=1.00 Hz, 1 H) 7.75 (dd, *J*=5.73, 3.28 Hz, 1 H) 8.09 (d, *J*=1.00 Hz, 1 H) 8.54 (s, 1 H) 8.60 (br s, 1 H)

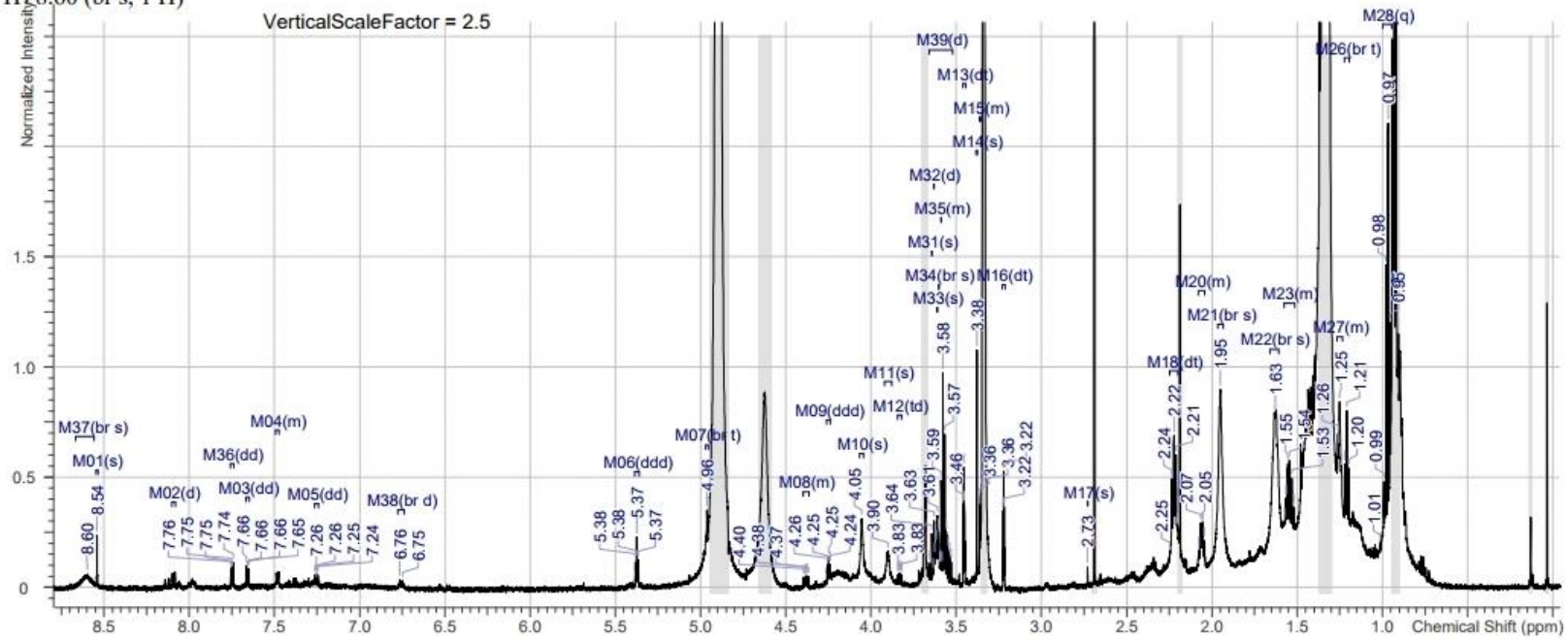

Figure A5: NMR data for compound 1, extracted using the solvent acetonitrile, and separated using TLC plate 1. This compound had an Rf value of 1 (See Table 3.9). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

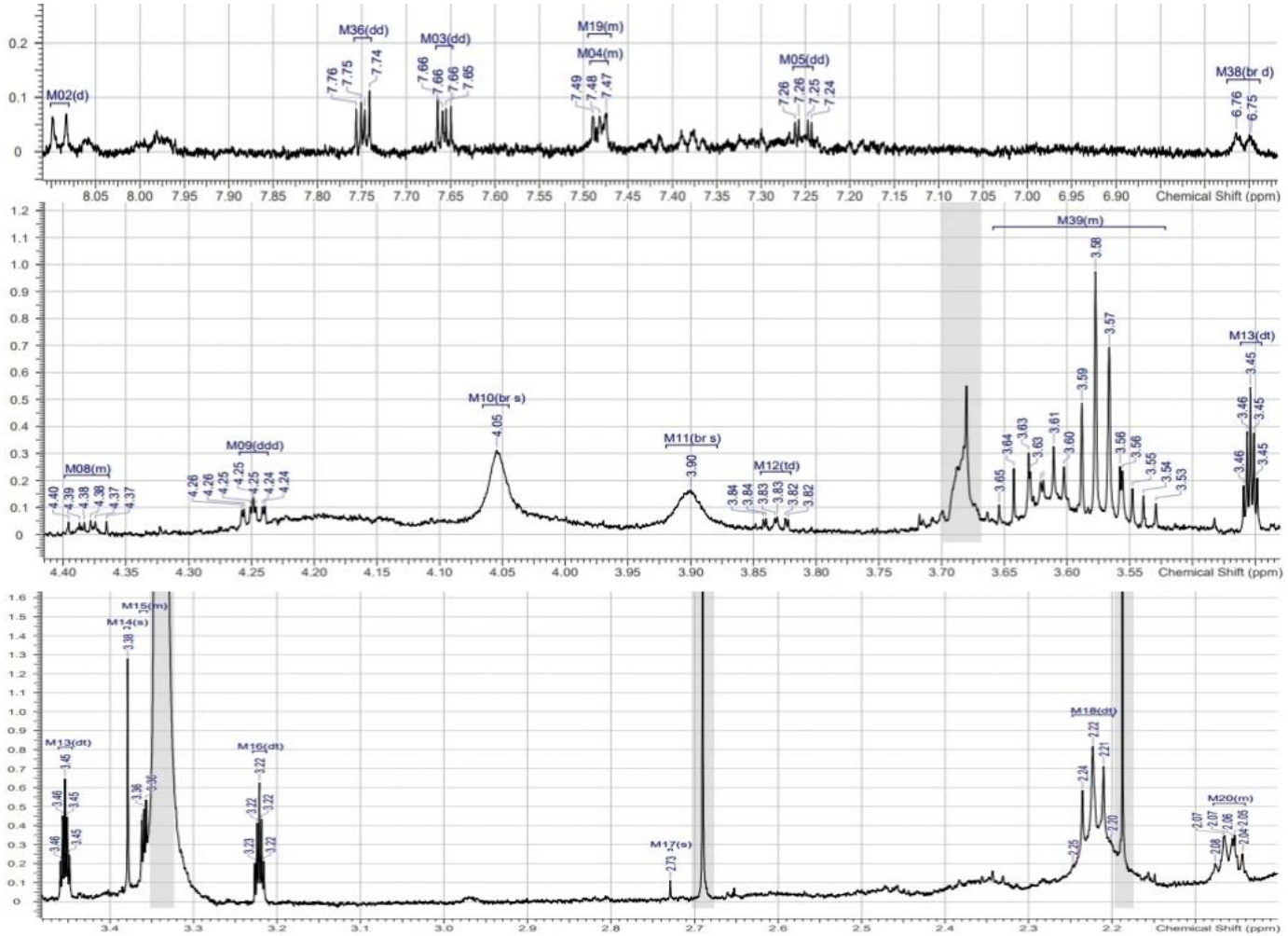

Figure A6: Supplementary NMR data for compound 1, extracted using the solvent acetonitrile, and separated using TLC plate 1. This compound had an Rf value of 1 (See Table 3.9). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

Acquisition Time (sec)	4.9633	**Comment**	5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079			**D**	0.06	**D1**	1
DE	12.93771	**DS**	2	**Date**	20 Jul 2017 15:08:35			**Date Stamp**	20 Jul 2017 15:08:35
File Name	C:\Users\Rob-W\Desktop\NMR Data\AcN Comp A\10\PDATA\1\1r					**Frequency (MHz)**	600.1300	**GB**	0
INSTRUM	<av600>	**LB**	0.2	**NS**	256	**Nucleus**	1H	**Number of Transients**	256
Origin	av600	**Original Points Count**	32768	**Owner**	nmrsu	**PC**	1		
PROBHD	<5 mm PATXI 1H/D-13C/15N Z-GRD Z855801/0079 >			**PULPROG**	<zg30>	**Points Count**	65536	**Pulse Sequence**	zg30
Receiver Gain	406.00	**SF**	600.129993527834			**SFO1**	600.13300065		
SI	65536	**SSB**	0	**SW(cyclical) (Hz)**	6602.11	**SWH**	6602.11267605634		
Solvent	METHANOL-d4			**Spectrum Offset (Hz)**	2989.1482	**Spectrum Type**	standard	**Sweep Width (Hz)**	6602.01
TD	65536	**TD0**	1	**TE**	297.96	**Temperature (degree C)**	24.960	**UNC1**	<1H>
WDW	1								

^{1}H NMR (600 MHz, METHANOL-d_4) δ ppm 0.87 - 0.92 (m, 25 H) 0.94 (q, *J*=7.55 Hz, 9 H) 1.18 (br t, *J*=1.00 Hz, 3 H) 1.21 (t, *J*=7.01 Hz, 7 H) 1.31 (s, 15 H) 1.41 (s, 1 H) 1.43 (ddd, *J*=1.00 Hz, 5 H) 1.52 (sxt, *J*=1.00 Hz, 6 H) 1.60 (br s, 10 H) 1.68 (quin, *J*=1.00 Hz, 2 H) 1.90 (s, 1 H) 2.30 - 2.36 (m, 1 H) 2.66 (s, 1 H) 2.70 (s, 1 H) 3.06 (s, 1 H) 3.19 (dt, *J*=3.29, 1.64 Hz, 1 H) 3.33 - 3.33 (m, 1 H) 3.35 (s, 1 H) 3.42 (dt, *J*=3.29, 1.65 Hz, 1 H) 3.49 - 3.61 (m, 6 H) 3.65 (s, 1 H) 3.79 (s, 1 H) 3.81 (s, 1 H) 3.85 (s, 1 H) 3.93 (s, 1 H) 4.01 (s, 1 H) 4.22 (ddd, *J*=5.65, 4.34, 1.15 Hz, 1 H) 4.29 (t, *J*=1.00 Hz, 1 H) 4.33 - 4.37 (m, 1 H) 4.59 (s, 2 H) 5.07 (d, *J*=1.24 Hz, 1 H) 5.92 (s, 1 H) 6.63 (d, *J*=8.43 Hz, 1 H) 6.81 (br d, *J*=8.89 Hz, 1 H) 6.96 (s, 1 H) 7.22 (dd, *J*=8.63, 2.54 Hz, 1 H) 7.44 - 7.46 (m, 1 H) 7.51 (br d, *J*=1.00 Hz, 1 H) 7.63 (dd, *J*=1.00 Hz, 1 H) 7.72 (dd, *J*=5.71, 3.28 Hz, 1 H) 8.09 (s, 1 H) 8.10 (s, 1 H) 8.11 (s, 1 H) 8.24 (s, 1 H) 8.51 (s, 1 H) 8.55 (br s, 1 H)

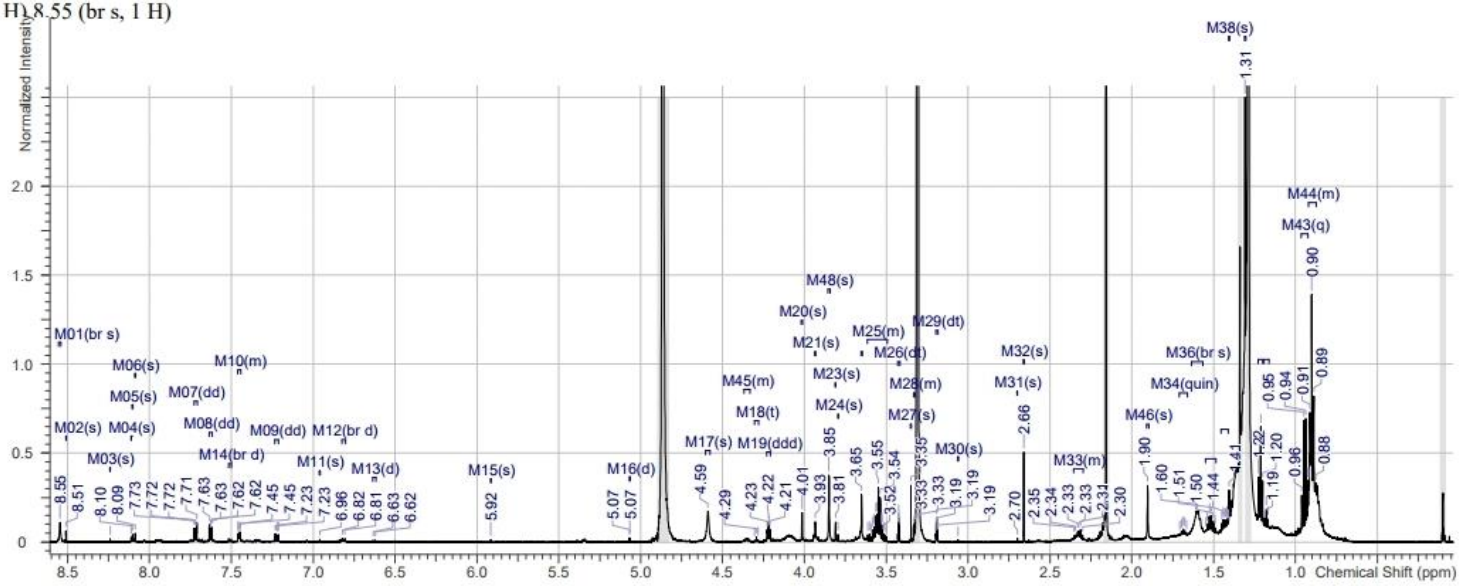

Figure A7: NMR data for compound 1, extracted using the solvent acetonitrile, and separated using TLC plate 2. This compound had an Rf value of 1 (See Table 3.9). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.

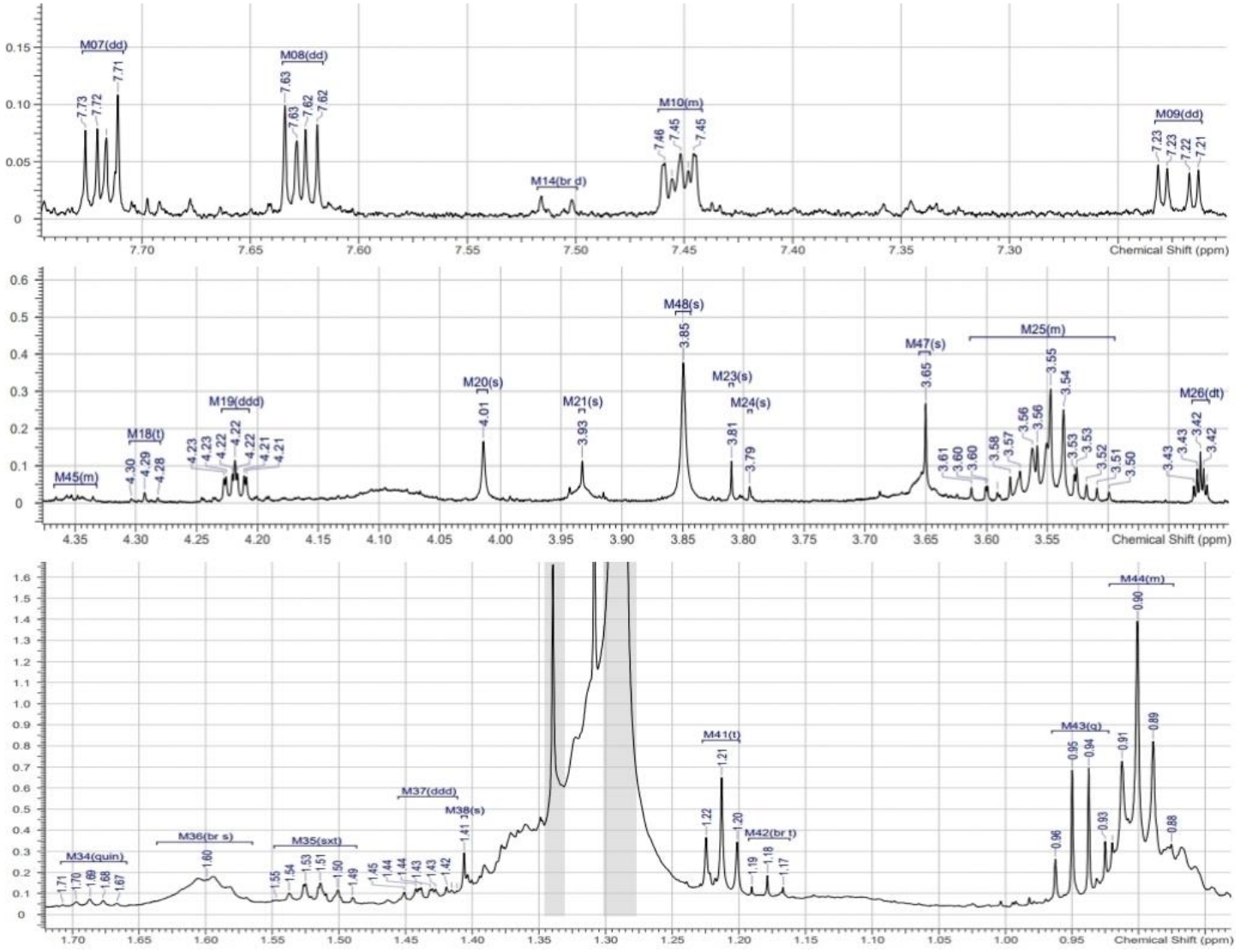

Figure A8: Supplementary NMR data for compound 1, extracted using the solvent acetonitrile, and separated using TLC plate 2. This compound had an Rf value of 1 (See Table 3.9). The work and data was collected by Dr Adam LeGresley and Mr Rob Warren.